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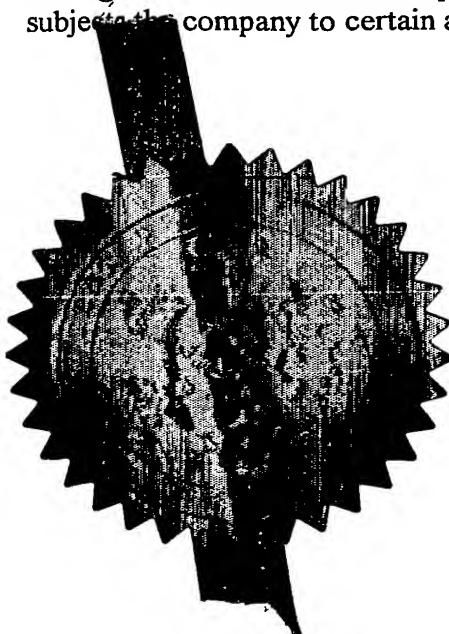
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Request for grant of a patent

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28 NOV 2003

1. Your reference

IS/CP6190060

2. Patent application number

(The Patent Office will fill this part in)

0327721.7

3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

BIOTICA TECHNOLOGY
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PFIZER INC.
235 East 42nd Street
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United States of America

Patents ADP number (*if you know it*)08763138001
GB00657833001
US

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

POLYKETIDES AND THEIR SYNTHESIS

5. Name of your agent (*if you have one*)

"Address for service" in the United Kingdom to which all correspondence should be sent
(*including the postcode*)

MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP

Patents ADP number (*if you know it*)

109006 ✓

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months.

Country

Priority application number
(*if you know it*)Date of filing
(day / month / year)

7. Divisionals, etc: Complete this section only if this application is a divisional application or resulted from an entitlement dispute (see note f)

Number of earlier UK application
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8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request?

YES

Answer YES if:

- a) any applicant named in part 3 is not an inventor, or
- b) there is an inventor who is not named as an applicant, or
- c) any named applicant is a corporate body.

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Patents Form 1/77

9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form	0
Description	49
Claim(s)	0
Abstract	0
Drawing(s)	24 + 24 //

10. If you are also filing any of the following, state how many against each item.

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	0
Request for a preliminary examination and search (Patents Form 9/77)	0
Request for a substantive examination (Patents Form 10/77)	0
Any other documents (please specify)	0

11. I/We request the grant of a patent on the basis of this application.

Signature(s)

Date 27 November 2003

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom

IAN STUART
0117 926 6411

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Polyketides and their synthesis

Field of Invention

The present invention relates to processes and materials (including recombinant strains) for the preparation and isolation of macrolide compounds, particularly compounds differing from natural compounds at least in terms of glycosylation. It is particularly concerned with erythromycin and azithromycin analogues wherein the natural sugar at the 5-position has been replaced. The invention includes the use of recombinant cells in which gene cassettes are expressed to generate novel macrolide antibiotics.

10

Background to the Invention

The biosynthetic pathways to the macrolide antibiotics produced by actinomycete bacteria generally involve the assembly of an aglycone structure, followed by specific modifications which may include any or all of: hydroxylation or other oxidative steps, methylation and glycosylation. In the case of the 14-membered macrolide erythromycin A these modifications consist of the specific hydroxylation of 6-deoxyerythronolide B to erythronolide B which is catalysed by EryF, followed by the sequential attachment of mycarose via the hydroxyl group at C-3 catalysed by the mycarosyltransferase EryBV (Staunton and Wilkinson, 1997). The attachment of desosamine via the hydroxyl group at C-5, catalysed by EryCIII, then results in the production of erythromycin D, the first intermediate with antibiotic activity. Erythromycin D is subsequently converted to erythromycin A by hydroxylation at C-12 (EryK) and O-methylation (EryG) on the mycarosyl group, this order being preferred (Staunton and Wilkinson, 1997). The biosynthesis of dTDP-L-mycarose and dTDP-D-desosamine has been studied in detail (Gaisser *et al.*, 1997; Summers *et al.*, 1997; Gaisser *et al.*, 1998; Salah-Bey *et al.*, 1998).

Recently 3.1 Å high-resolution X-ray investigation of the interaction of ribosomes with macrolides (Schlünzen *et al.*, 2001, Hansen *et al.*, 2002) has revealed key interactions giving direct insights into ways in which macrolide templates might be adapted, by chemical or biological approaches, for increased ribosomal binding and inhibition and for improved effectiveness against resistant organisms. In particular, previous indications about the importance of the sugar substituent at the C-5 hydroxyl of the macrocycle for ribosomal binding are fully borne out by the structural analysis; this substituent extends towards the peptidyl transferase centre and in the case of 16 membered macrolides, which bear a disaccharide at C-5, reaches further into the peptidyl transferase centre, thus providing a molecular basis for the observation that 16 membered macrolides inhibit ribosomal capacity

to form even a single peptide bond (Poulsen *et al.*, 2000). This suggests that erythromycins with alternative substituents at the C-5 positions, for example mycaminosyl and angulosaminosyl erythromycins, and in particular mycaminosyl and 4'-*O* substituted mycaminosyl erythromycins, are highly desirable as potential anti-bacterial agents.

5 Since post-polyketide synthase modifications are often critical for biological activity (Liu and Thorson, 1994; Kaneko *et al.*, 2000), there has been increasing interest in understanding the mechanism and specificity of the enzymes involved to engineer the biosynthesis of diverse novel hybrid macrolides with potentially improved activities. Recent work has demonstrated that the manipulation of sugar biosynthetic genes is a powerful approach to isolate novel macrolide antibiotics. The recently demonstrated relaxed specificity 10 of the glycosyltransferases is crucial for this approach (see Méndez and Salas, 2001 and references therein). In the pathways to erythromycin A and methymycin / neomethymycin, the production of hybrid macrolides has been observed after inactivation of specific genes involved in the biosynthesis of deoxyhexoses (Gaisser *et al.*, 1997; Summers *et al.*, 1997; Gaisser *et al.*, 1998; Salah-Bey *et al.*, 1998; Zhao *et al.*, 1998a; Zhao *et al.*, 1998b) or after 15 the expression of genes from different biosynthetic gene clusters (Zhao *et al.*, 1999). A relaxed specificity towards the sugar substrate has also been reported for glycosyltransferases that have been expressed in heterologous strains, including glycosyltransferases from the pathways to vancomycin (Solenberg *et al.*, 1997), elloramycin (Wohlert *et al.*, 1998), oleandomycin (Doumith *et al.*, 1999; Gaisser *et al.*, 2000), pikromycin (Tang and McDaniel, 2001), epirubicin (Madduri *et al.*, 1998), avermectin (Wohlert *et al.*, 2001) and spinosyn (Gaisser *et al.*, 2002a). Most of the successful alterations so far reported have involved 20 relaxed specificity towards the activated sugar moiety, while as yet only isolated examples are known where a glycosyltransferase targets its deoxysugar to an alternative aglycone substrate (Spagnoli *et al.*, 1983; Trefzer *et al.*, 1999). Both WO 97/23630 and WO 99/05283 describe 25 the production of erythromycins with an altered glycosylation pattern in culture supernatants by deletion of a specific sugar biosynthesis gene. Thus WO 99/05283 describes low but detectable levels of 5-*O*-dedesosaminyl-5-*O*-mycaminosyl erythromycin D in the culture supernatant of an *eryCIV* knockout strain of *S. erythraea*. It also has been demonstrated that 30 the use of the gene cassette technology described in patent WO01/79520 is a powerful and potentially general approach to isolate novel macrolide antibiotics by expressing combinations of genes in mutant strains of *S. erythraea* (Gaisser *et al.*, 2002b). WO 01/79520 also describes the detection of 5-*O*-dedesosaminyl-5-*O*-mycaminosyl erythromycin A in culture supernatants of the *S. erythraea* strains SGQ2pSGCIII and SGQ2p(mycaminose)CIII, 35 fed with 3-*O*-mycarosyl erythronolide B. However, the low levels of 5-*O*-dedesosaminyl-5-*O*-

mycaminosyl erythromycin A make this a less than optimal method for producing this valuable material on large scales and similar problems were encountered synthesizing 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A using chemical methods (Jones *et al.*, 1969). EP 1024145 refers to the isolation of azithromycin analogues carrying a mycaminosyl residue such as 5-O-dedesosaminyl-5-O-mycaminosyl azithromycin and 3"-desmethyl-5-O-dedesosaminyl-5-O-mycaminosyl azithromycin. However the only examples given in this area are "prophetic examples" and there is no evidence that they could actually be put into practice.

Therefore, the present invention provides the first demonstration of an efficient and highly effective method for making significant quantities of erythromycins and azithromycins which have non-natural sugars at the C-5 position, in particular mycaminose and angulosamine. In a specific aspect the present invention provides for the synthesis of mycaminose and angulosamine using specific combinations of sugar biosynthetic genes in gene cassettes.

15

Summary of the Invention

The present invention relates to processes, and recombinant strains, for the preparation and isolation of erythromycins and azithromycins, which differ from the corresponding naturally occurring compound in the glycosylation of the C-5 position. In particular, the present invention relates to processes and recombinant strains for the preparation and isolation of 5-O-dedesosaminyl-5-O-mycaminosyl, or angulosaminyl erythromycins and azithromycins, in particular 5-O-dedesosaminyl-5-O-mycaminosyl erythromycins and 5-O-dedesosaminyl-5-O-mycaminosyl azithromycins, and specifically 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin C, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin D, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A, and 5-O-dedesosaminyl-5-O-mycaminosyl azithromycin. The present invention further relates to novel 5-O-dedesosaminyl-5-O-mycaminosyl, angulosaminyl erythromycins and azithromycins produced thereby.

30

Detailed description of the Invention

The present invention relates to processes, and recombinant strains, for the preparation and isolation of erythromycins and azithromycins which differ from the naturally occurring compound in the glycosylation of the C-5 position. These are referred to herein as "compounds of the invention" and unless the context dictates otherwise, such a reference includes a reference to 5-O-dedesosaminyl-5-O-mycaminosyl erythromycins, 5-O-

dedesosaminyl-5-O-angolosaminyl erythromycins, 5-O-dedesosaminyl-5-O-mycaminosyl azithromycins, and 5-O-dedesosaminyl-5-O-angolosaminyl azithromycins, specifically 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin C, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin D, 5-O-dedesosaminyl-5-O-mycaminosyl azithromycin, 5-O-dedesosaminyl-5-O-angolosaminyl erythromycin A, 5-O-dedesosaminyl-5-O-angolosaminyl erythromycin B, 5-O-dedesosaminyl-5-O-angolosaminyl erythromycin C, 5-O-dedesosaminyl-5-O-angolosaminyl erythromycin D, 5-O-dedesosaminyl-5-O-angolosaminyl azithromycin and analogues thereof which additionally vary in glycosylation at the C3 position (see WO 01/79520) and which may also vary in the aglycone backbones (see WO 98/01571, EP 1024145, WO 93/13663, WO 98/49315). The invention relates to processes, and recombinant strains, for the preparation and isolation of compounds of the invention. The present invention further relates to novel 5-O-dedesosaminyl-5-O-angolosaminyl erythromycins and azithromycins produced thereby (Figure 1). The methodology comprises in part the expression of a gene cassette in the *S. erythraea* mutant strain SGQ2 (which carries genomic deletions in *eryA*, *eryCIII*, *eryBV* and *eryCIV* (WO01/79520)), as described in Example 3 and 6 and in *S. erythraea* Q42/1 (BIOT-2166) (Examples 1- 4) and *S. erythraea* 18A1 (BIOT-2634) (Example 6). Detailed descriptions are given in Examples 1 - 11.

The invention relates to a process involving the transformation of an actinomycete strain, including but not limited to strains of *S. erythraea* such as SGQ2, (see WO 01/79520) or Q42/1 or 18A1 (whose preparation is described below) with an expression plasmid containing a combination of genes which are able to direct the biosynthesis of a sugar moiety and direct its subsequent transfer to an aglycone or pseudoaglycone.

In a particular embodiment the present invention relates to a gene cassette containing a combination of genes which are able to direct the synthesis of mycaminose in an appropriate strain background. The gene cassette may include genes selected from but not limited to *angorf14*, *tylMIII*, *tylMI*, *tylB*, *tylAI*, *tylAII*, *tylIa*, *angAI*, *angAII*, *angMIII*, *angB*, *angMI*, *eryG*, *eryK* and glycosyltransferase genes including but not limited to *tylMII*, *angMII*, *desVII*, *eryCIII*, *eryBV*, *spnP*, and *midI*. In a preferred embodiment the gene cassette comprises *angorf14* in combination with one or more other genes which are able to direct the synthesis of mycaminose. In a more preferred embodiment the gene cassette comprises *angAI*, *angAII*, *angorf14*, *angMIII*, *angB*, *angMI*, in combination with one or more glycosyltransferases such as but not limited to *eryCIII*, *tylMII*, *angMII*. In an alternative embodiment the gene cassette comprises *tylAI*, *tylAII*, *tylMIII*, *tylB*, *tylIa*, *tylMI* in

combination with glycosyltransferases such as but not limited to *eryCIII*, *tylMII* and *angMII*. In a preferred embodiment the strain is an *S. erythraea* strain.

In a particular embodiment the present invention relates to a gene cassette containing combinations of genes which are able to direct the synthesis of angulosamine, including but not limited to *angMIII*, *angM1*, *angB*, *angA1*, *angAII*, *angorf14*, *angorf4*, *tylMIII*, *tylM1*, *tylB*, *tylA1*, *tylAII*, *eryCVI*, *spnO*, *eryBVI*, and *eryK* and one or more glycosyltransferase genes including but not limited to *eryCIII*, *tylMII*, *angMII*, *desVII*, *eryBV*, *spnP* and *midI*. In a preferred embodiment the gene cassette contains *angMIII*, *angM1*, *angB*, *angA1*, *angAII*, *angorf14*, *spnO* in combination with a glycosyltransferase gene such as but not limited to *angMII*, *tylMII* or *eryCIII*. In a preferred embodiment the strain is an *S. erythraea* strain.

In one embodiment, the process of the present invention further involves feeding of an aglycone and/or a pseudoaglycone substrate (for definition see below), including (but not limited to) 3-*O*-mycarosyl erythronolide B, erythronolide B, 6-deoxy erythronolide B, 3-*O*-mycarosyl-6-deoxy erythronolide B, tylactone, spinosyn pseudoaglycone, 3-*O*-rhamnosyl erythronolide B, 3-*O*-rhamnosyl-6-deoxy erythronolide B to cultures of the transformed actinomycete strains, the bioconversion of the substrate to compounds of the invention and optionally the isolation of said compounds. This process is exemplified in Examples 1-11. However, a person of skill in the art will appreciate that in an alternative embodiment the host cell can express the desired aglycone template, either naturally or recombinantly.

As used herein, the term "pseudoaglycone" refers to a partially glycosylated intermediate of a multiply-glycosylated product.

Those skilled in the art will appreciate that alternative host strains can be used. A preferred cell is a prokaryote or a fungal cell or a mammalian cell. A particularly preferred host cell is a prokaryote, more preferably host cell strains such as actinomycetes, *Pseudomonas*, myxobacteria, and *E. coli*. It will be appreciated that if the host cell does not naturally produce erythromycin, or a closely related 14-membered macrolide, it may be necessary to introduce a gene conferring self-resistance to the macrolide product, such as *ermE* from *S. erythraea*. Even more preferably the host cell is an actinomycete, even more preferably strains that include but are not limited to *S. erythraea*, *Streptomyces griseofuscus*, *Streptomyces cinnamonensis*, *Streptomyces albus*, *Streptomyces lividans*, *Streptomyces hygroscopicus* sp., *Streptomyces hygroscopicus* var. *ascomyceticus*, *Streptomyces longisporoflavus*, *Saccharopolyspora spinosa*, *Streptomyces tsukubaensis*, *Streptomyces coelicolor*, *Streptomyces fradiae*, *Streptomyces rimosus*, *Streptomyces avermitilis*, *Streptomyces eurythermus*, *Streptomyces venezuelae*, *Amycolatopsis mediterranei*. In a more highly preferred embodiment the host cell is *S. erythraea*.

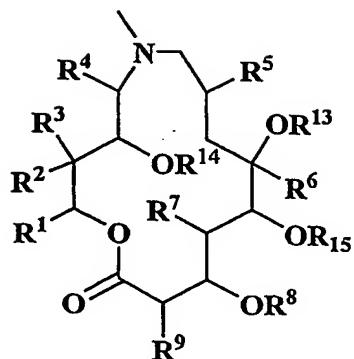
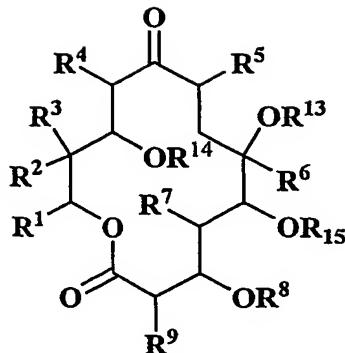
It will readily occur to those skilled in the art that the substrate fed to the recombinant cultures of the invention need not be a natural intermediate in erythromycin biosynthesis. Thus, the substrate could be modified in the aglycone backbone (see Examples 8-11) or in the sugar attached at the 3-position or both. WO 01/79520 demonstrates that the desosaminyl transferase EryCIII exhibits relaxed specificity with respect to the pseudoaglycone substrate, converting 3-*O*-rhamnosyl erythronolides into the corresponding 3-*O*-rhamnosyl erythromycins. Appropriate modified substrates may also be produced by chemical semi-synthetic methods. Alternatively, methods of engineering the erythromycin-producing polyketide synthase, DEBS, to produce modified erythromycins are well known in the art (for example WO 93/13663, WO 98/01571, WO 98/01546, WO 98/49315, Kato, Y. et al., 2002). Likewise, WO 01/79520 describes methods for obtaining erythronolides with alternative sugars attached at the 3-position. Therefore, the term "compounds of the invention" includes all such non-natural aglycone compounds as described previous additionally with alternative sugars at the C-5 position. All these documents are incorporated herein by reference.

It will readily occur to those skilled in the art that the compounds of the invention containing a mycaminosyl moiety at the C-5 position could be modified at the C4 hydroxyl group of the mycaminosyl moiety, including but not limited to glycosylation (see also WO 01/79520), acylation or chemical modification.

The present invention thus provides variants of erythromycin and related macrolides having at the 5-position a non-naturally occurring sugar, in particular an *O*-mycaminosyl, or angulosaminyl residue or a derivative or precursor thereof, specifically an *O*- angulosaminyl residue or a derivative thereof.

The term "variants of erythromycin" encompasses (a) erythromycins A, B, C and D; (b) semi-synthetic derivatives such as azithromycin and other derivatives as discussed in EP 1024145, which is incorporated herein by reference; (c) variants produced by genetic engineering and semi-synthetic derivatives thereof. Variants produced by genetic engineering include variants as taught in, or producible by, methods taught in WO 98/01571, EP 1024145, WO 93/13663, WO 98/49315 and WO 01/79520 which are incorporated herein by reference. The compounds of the invention include variants of erythromycin where the natural sugar at position C5 has been replaced with mycaminoose or angulosamine and also includes compounds of the following formula (1) and pharmaceutically acceptable salts thereof. No stereochemistry is shown in Formula 1 as all possibilities are covered, including "natural" stereochemistries (as shown elsewhere in this specification) at some or all positions.

35 Formula I:

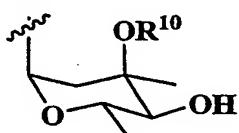


$R^1 = H, CH_3, C_2H_5$ or selected from i) see below

R^2, R^4, R^5, R^6, R^7 and R^9 are each independently H, OH, CH_3, C_2H_5 or OCH_3

$R^3 = H$ or OH

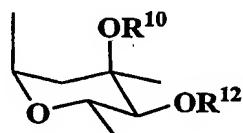
$R^8 = H$ or



or selected from iv) see below

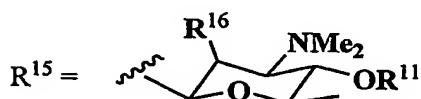
$R^{10} = H$ or CH_3 or acyl

$R^{11} = H$ or



$R^{12} = H$ or acyl

$R^{13} = H$ or CH_3



$R^{16} = H$ or OH

$R^{14} = H$ or $-C(O)NR^cR^d$ wherein each of R^c and R^d is independently H, C_1-C_{10} alkyl, C_2-C_{20} alkenyl, C_2-C_{10} alkynyl, $-(CH_2)_m(C_6-C_{10}$ aryl), or $-(CH_2)_m(5-10$ membered heteroaryl), wherein m is an integer ranging from 0 to 4, and wherein each of the foregoing R^c and R^d

5 groups, except H , may be substituted by 1 to 3 Q groups; or wherein R^c and R^d may be taken together to form a 4-7 membered saturated ring or a 5-10 membered heteroaryl ring, wherein said saturated and heteroaryl rings may include 1 or 2 heteroatoms selected from O, S and N, in addition to the nitrogen to which R^c and R^d are attached, and said saturated ring may

- include 1 or 2 carbon-carbon double or triple bonds, and said saturated and heteroaryl rings may be substituted by 1 to 3 Q groups; or R² and R¹⁷ taken together form a carbonate ring; each Q is independently selected from halo, cyano, nitro, trifluoromethyl, azido, -C(O)Q¹, -OC(O)Q¹, -C(O)OQ¹, -OC(O)OQ¹, -NQ²C(O)Q³, -C(O)NQ²Q³, -NQ²Q³, hydroxy, C₁-C₆
- 5 alkyl, C₁-C₆ alkoxy, -(CH₂)_m(C₆-C₁₀ aryl), and -(CH₂)_m(5-10 membered heteroaryl), wherein m is an integer ranging from 0 to 4, and wherein said aryl and heteroaryl substituents may be substituted by 1 or 2 substituents independently selected from halo, cyano, nitro, trifluoromethyl, azido, -C(O)Q¹, -C(O)OQ¹, -OC(O)OQ¹, -NQ²C(O)Q³, -C(O)NQ²Q³, -NQ²Q³, hydroxy, C₁-C₆ alkyl, and C₁-C₆ alkoxy;
- 10 each Q¹, Q² and Q³ is independently selected from H, OH, C₁-C₁₀ alkyl, C₁-C₆ alkoxy, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, -(CH₂)_m(C₆-C₁₀ aryl), and -(CH₂)_m(5-10 membered heteroaryl), wherein m is an integer ranging from 0 to 4; with the proviso that the compound is not 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A or D.
- The present invention also provides compounds according to formula I above in which:
- 15 i) the substituent R¹ is selected from
- an alpha-branched C₃-C₈ group selected from alkyl, alkenyl, alkynyl, alkoxyalkyl and alkylthioalkyl groups any of which may be optionally substituted by one or more hydroxyl groups;
 - a C₅-C₈ cycloalkylalkyl group wherein the alkyl group is an alpha-branched C₂-C₅ alkyl group
 - a C₃-C₈ cycloalkyl group or C₅-C₈ cycloalkenyl group, either of which may optionally be substituted by one or more hydroxyl, or one or more C₁-C₄ alkyl groups or halo atoms
 - a 3 to 6 membered oxygen or sulphur containing heterocyclic ring which may be saturated, or fully or partially unsaturated and which may optionally be substituted by one or more C₁-C₄ alkyl groups, halo atoms or hydroxyl groups.
 - phenyl which may be optionally substituted with at least one substituent selected from C₁-C₄ alkyl, C₁-C₄ alkoxy and C₁-C₄ alkylthio groups, halogen atoms, trifluoromethyl, and cyano or
- 20
- 25
- 30
- 35
- R¹ is R¹⁷-CH₂- where R¹⁷ is H, C₁-C₈ alkyl, C₂-C₈ alkenyl, C₂-C₈ alkynyl, alkoxyalkyl or alkylthioalkyl containing from 1 to 6 carbon atoms in each alkyl or alkoxy group wherein any of said alkyl, alkoxy, alkenyl or alkynyl groups may be substituted by one or more hydroxyl groups or by one or more halo atoms; or a C₃-C₈ cycloalkyl or C₅-C₈ cycloalkenyl either of which may be optionally substituted by one or more C₁-C₄ alkyl groups or halo atoms; or

- 9
- a 3 to 6 membered oxygen or sulphur containing heterocyclic ring which may be saturated or fully or partially unsaturated and which may optionally be substituted by one or more C₁-C₄ alkyl groups or halo atoms; or a group of the formula SA₁₆ wherein A₁₆ is C₁-C₈ alkyl, C₂-C₈ alkenyl, C₂-C₈ alkynyl, C₃-C₈ cycloalkyl, C₅-C₈ cycloalkenyl, phenyl or substituted phenyl wherein the substituent is C₁-C₄ alkyl, C₁-C₄ alkoxy or halo, or a 3 to 6 membered oxygen or sulphur-containing heterocyclic ring which may be saturated, or fully or partially unsaturated and which may optionally be substituted by one or more C₁-C₄ alkyl groups or halo atoms
- 5 ii) the substituents R², R⁴, R⁵, R⁶, R⁷ and R⁹ are each, independently, H, OH, CH₃, C₂H₅, OCH₃
- 10 iii) the -CHOH- at C11 (erythromycins) or C12 (azithromycins) is replaced by a methylene group (-CH₂-), a keto group (C=O), or by a 10,11-olefinic bond (erythromycins) or 11,12-olefinic bond (azithromycins)
- 15 iv) R⁸ includes but is not limited to rhamnose, 2'-O-methyl rhamnose, 2',3'-bis-O-methyl rhamnose, 2',3',4'-tri-O-methyl rhamnose, oleandrose, olioose, digitoxose or olivose
- 20 v) the substituent R¹¹ is H or mycarose or C4-O-acyl-mycarose or glucose
- The present invention also provides compounds according to formula I above which differ in the oxidation state of one or more of the ketide units (i.e. selection of alternatives from the group: -CO-, -CH(OH)-, alkene -CH-, and CH₂) where the stereochemistry of any -CH(OH)- is also independently selectable.
- 25 Novel 5-O-dedesosaminy1-5-O-angulosaminy1 erythromycins and azithromycins made available by this aspect of the invention include, but are not limited to those where in the R¹⁵ group R¹¹ = R¹⁶ = H, with the proviso that they are not angolamycin or medermycin (Kinumaki and Suzuki, 1972; Ichinose *et al.*, 2003).
- 30 Additionally, a person of skill in the art will appreciate that using the methods of the present invention mycaminoose and angulosamine may be added to other aglycones or pseudoaglycone for example (but without limitation) tylactone or spinosyn pseudoaglycone. These other aglycones or pseudoaglycones may be the naturally occurring structure or they may be modified in the aglycone backbone, such modified substrates may be produced by chemical semi-synthetic methods (Kaneko *et al.*, 2000 and references cited therein). or, alternatively, via PKS engineering, such methods are well known in the art (for example WO 93/13663, WO 98/01571, WO 98/01546, WO 98/49315, Kato, Y. *et al.*, 2002)).

Moreover, the process of the host cell selection further comprises the optional step of deleting or inactivating or adding or manipulating genes in the host cell. This process comprises the improvement of recombinant host strains for the preparation and isolation of compounds of the invention, in particular 5-O-dedesosaminyl-5-O-mycaminosyl erythromycins and 5-O-dedesosaminyl-5-O-mycaminosyl azithromycins, specifically 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin C, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B, 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin D and 5-O-dedesosaminyl-5-O-mycaminosyl azithromycin. This approach is exemplified in Example 1 by introducing an *eryBVT* mutation into the 10 chromosome of *S. erythraea* SGQ2 in order to optimise the conversion of the substrate 3-O-mycarosyl erythronolide B to 5-O-dedesosaminyl-5-O-mycaminosyl erythromycins.

In a further aspect the invention relates to the construction of gene cassettes. The cloning method used to isolate these gene cassettes is analogous to that used in PCT/GB03/003230 and diverges significantly from the approach previously described (WO 15 01/79520) by assembling the gene cassette directly in an expression vector rather than pre-assembling the genes in pUC18/19 plasmids, thus providing a more rapid cloning procedure for the isolation of gene cassettes. The strategy for isolating these gene cassettes is exemplified in Example 1 to Example 11. A schematic overview of the strategy is given in Figure 2.

Another aspect of the invention allows the enhancement of gene expression by changing the order of genes in a gene cassette, the genes including but not limited to *tylMI*, *tylMIII*, *tylB*, *eryCVI*, *tylAI*, *tylAII*, *eryCIII*, *eryBV*, *angAI*, *angAII*, *angMIII*, *angB*, *angM1*, *angorf14*, *angorf4*, *eryBVI*, *eryK*, *eryG*, *angMII*, *tylMII*, *desVII*, *midI*, *spnO*, *spnN*, *spnP* and genes with similar functions, allowing the arrangement of the genes in a multitude of permutations (Figure 2).

The cloning strategy outlined in this invention also allows the introduction of a histidine tag in combination with a terminator sequence 3' of the gene cassette to enhance gene expression (see Example 1). Those skilled in the art will appreciate other terminator sequences well known in the art could be used. See, for example Bussiere and Bastia (1999), Bertram *et al.* (2001) and Kieser *et al.* (2000), incorporated herein by reference.

Another aspect of the invention comprises the use of alternative promoters such as *ptipA* (Ali *et al.*, 2002) and/or *ptr* (Salah-Bey *et al.*, 1995) to express genes and/or assembled gene cassette(s) to enhance expression.

Another aspect of the invention describes the multiple uses of promoter sequences in the assembled gene cassette to enhance gene expression as exemplified in Example 6.

Another aspect of the invention describes the addition of genes encoding for a NDP-glucose-synthase such as *tylAI* and a NDP-glucose-4,6-dehydratase such as *tylAII* to the gene cassette in order to enhance the endogenous production of the activated sugar substrate. Those skilled in the art will appreciate that alternative sources of equivalent sugar biosynthetic pathway genes may be used. In this context alternative sources include but are not limited to:

5 TylAI- homologues: DesIII of *Streptomyces venezuelae* (accession no AAC68682), GrsD of *Streptomyces griseus* (accession no AAD31799), AveBIII of *Streptomyces avermitilis* (accession no BAA84594), Gtt of *Saccharopolyspora spinosa* (accession no AAK83289), SnogJ of *Streptomyces nogalater* (accession no AAF01820), AclY of 10 *Streptomyces galilaeus* (accession no BAB72036), LanG of *Streptomyces cyanogenus* (accession no AAD13545), Graorf16(GraD) of *Streptomyces violaceoruber* (accession no AAA99940), OleS of *Streptomyces antibioticus* (accession no AAD55453) and StrD of *Streptomyces griseus* (accession no A26984) and AngAI of *S. eurythermus*.

15 TylAII- homologues: AprE of *Streptomyces tenebrarius* (accession no AAG18457), GdH of *S. spinosa* (accession no AAK83290), DesIV of *S. venezuelae* (accession no AAC68681), GdH of *S. erythraea* (accession no AAA68211), AveBII of *S. avermitilis* (accession no BAA84593), Scf81.08C of *Streptomyces coelicolor* (accession no CAB61555), LanH of *S. cyanogenus* (accession no AAD13546), 20 Graorf17 (GraE) of *S. violaceoruber* (accession no S58686), OleE of *S. antibioticus* (accession no AAD55454), StrE of *S. griseus* (accession no P29782) and AngAII of *S. eurythermus*.

25 Similarly, alternative sources for activated sugar biosynthesis gene homologues to *tylMIII*, *angAIII*, *eryCII*, *tylMII*, *angMII*, *tylB*, *angB*, *eryCI*, *tylMI*, *angMI*, *eryCVI*, *tylla*, *angorf14*, *angorf4*, *spnO*, *eryBVI*, *eryBV*, *eryCIII*, *desVII*, *midI*, *spnN* and *spnP* will readily occur to those skilled in the art, and can be used.

30 Another aspect of the invention describes the use of alternative glycosyltransferases in the gene cassettes such as EryCIII. Those skilled in the art will appreciate that alternative glycosyltransferases may be used. In this context alternative glycosyltransferases include but are not limited to: TylMII (Accession no CAA57472), DesVII (Accession no AAC68677), MegCIII (Accession no AAG13921), MegDI (Accession no AAG13908) or AngMII of *S. eurythermus*.

35 In one aspect of the present invention, the gene cassette may additionally comprise a chimeric glycosyltransferase (GT). This is particularly of benefit where the natural GT does not recognise the combination of sugar and aglycone that is required for the synthesis of the

desired analogue. Therefore, in this aspect the present invention specifically contemplates the use of a chimeric GT wherein part of the GT is specific for the recognition of the sugar whose synthesis is directed by the genes in said expression cassette when expressed in an appropriate strain background and part of the GT is specific for the aglycone or

5 pseudoaglycone template (Hu and Walker, 2002).

Those skilled in the art will appreciate that different strategies may be used for the introduction of gene cassettes into the host strain, such as site-specific integration vectors (Smovkina *et al.*, 1990; Lee *et al.*, 1991; Matsuura *et al.*, 1996; Van Mellaert *et al.*, 1998; Kieser *et al.*, 2000). Alternatively, plasmids containing the gene cassettes may be integrated

10 into any neutral site on the chromosome using homologous recombination sites. Further, for a number of actinomycete host strains, including *S. erythraea*, the gene cassettes may be introduced on self-replicating plasmids (Kieser *et al.*, 2000; WO 98/01571).

A further aspect of the invention provides a process for the production of compounds of the invention and optionally for the isolation of said compounds.

15 A further aspect of the invention is the use of different fermentation methods to optimise the production of the compounds of the invention as exemplified in Example 1. Another aspect of the invention is the addition of *ery* genes such as *eryK* and/or *eryG* into the gene cassette. One skilled in the art will appreciate that the process can be optimised for the production of a specific erythromycin (i.e. A, B, C, D) or azithromycin by manipulation of the

20 genes *eryG* (responsible for the methylation on the mycarose sugar) and/or *eryK* (responsible for hydroxylation at C12). Thus, to optimise the production of the A-form, an extra copy of *eryK* may be included into the gene cassette. Conversely, if the erythromycin B analogue is required, this can be achieved by deletion of the *eryK* gene from the *S. erythraea* host strain, or by working in a heterologous host in which the gene and/or its functional homologue, is

25 not present. Similarly, if the erythromycin D analogue is required, this can be achieved by deletion of both *eryG* and *eryK* genes from the *S. erythraea* host strain, or by working in a heterologous host in which both genes and/or their functional homologues are not present. Similarly, if the erythromycin C analogue is required, this can be achieved by deletion of the

30 *eryG* gene from the *S. erythraea* host strain, or by working in a heterologous host in which the gene and/or its functional homologues are not present.

In this context a preferred host cell strain is a mammalian cell strain, fungal cells strain or a prokaryote. More preferably the host cell strain is *Pseudomonas*, myxobacteria or *E. coli*. In a more preferred embodiment the host cell strain is an actinomycete, still more preferably including, but not limited to *Saccharopolyspora erythraea*, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseofuscus*, *Streptomyces*

cinnamomensis, *Streptomyces fradiae*, *Streptomyces eurythermus*, *Streptomyces longisporoflavus*, *Streptomyces hygroscopicus*, *Saccharopolyspora spinosa*, *Micromonospora griseorubida*, *Streptomyces lasaliensis*, *Streptomyces venezuelae*, *Streptomyces antibioticus*, *Streptomyces lividans*, *Streptomyces rimosus*, *Streptomyces albus*, *Amycolatopsis mediterranei*, *Nocardia* sp., *Streptomyces tsukubaensis* and *Actinoplanes* sp. N902-109. In a still more preferred embodiment the host cell strain is selected from *Saccharopolyspora erythraea*, *Streptomyces griseofuscus*, *Streptomyces cinnamomensis*, *Streptomyces albus*, *Streptomyces lividans*, *Streptomyces hygroscopicus* sp., *Streptomyces hygroscopicus* var. *ascomyceticus*, *Streptomyces longisporoflavus*, *Saccharopolyspora spinosa*, *Streptomyces tsukubaensis*, *Streptomyces coelicolor*, *Streptomyces fradiae*, *Streptomyces rimosus*, *Streptomyces avermitilis*, *Streptomyces eurythermus*, *Streptomyces venezuelae*, *Amycolatopsis mediterranei*. In the most highly preferred embodiment the host strain is *Saccharopolyspora erythraea*.

The present invention provides methods for the production and isolation of compounds of the invention, in particular of erythromycin and azithromycin analogues which differ from the natural compound in the glycosylation of the C-5 position, for example but without limitation: novel 5-*O*-dedesosaminyl-5-*O*-mycaminosyl or angulosaminyl erythromycins and 5-*O*-dedesosaminyl-5-*O*-mycaminosyl, or angulosaminyl azithromycins which are useful as anti-microbial agents for use in human or animal health.

In further aspects the present invention provides novel products as obtainable by any of the processes disclosed herein.

Brief description of Figures

Figure 1A: Structures of 5-*O*-dedesosaminyl-5-*O*-mycaminosyl erythromycin A, 5-*O*-dedesosaminyl-5-*O*-mycaminosyl erythromycin B and 5-*O*-dedesosaminyl-5-*O*-mycaminosyl erythromycin C.

Figure 1B: Structure of 5-*O*-dedesosaminyl-5-*O*-mycaminosyl azithromycin.

Figure 2: Schematic overview over the gene cassette cloning strategy. Vector pSG144 was derived from vector pSG142 (Gaisser *et al.*, 2000). Abbreviations: *dam*: DNA isolated from *dam* strain background, *Xba*I^{met}: *Xba*I site sensitive to Dam methylation, *ery*RHS: DNA fragment of the right hand side of the *ery*-cluster as described previously (Gaisser *et al.*, 2000).

Figure 3: Amino acid comparison between the published sequence of TylA1 (below) and the amino acid sequence detected from the sequencing data described in this invention (above). The changes in the amino acid sequence are underlined.

5 **Figure 4:** Amino acid comparison between the published sequence of TylAII (below) and the amino acid sequence detected from the sequencing data described in this invention (above). The changes in the amino acid sequence are underlined.

Figure 5: Structure of 5-O-angulosaminylyl tylactone.

10

Figure 6: Shows an overview of the angolamycin polyketide synthase gene cluster.

Figure 7: The DNA sequence which comprises *orf14* and *orf15 (angB)* from the angolamycin gene cluster.

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Figure 8: The DNA sequence which comprises *orf2 (angA1)*, *orf3 (angAII)* and *orf4* from the angolamycin gene cluster.

20

Figure 9: The DNA sequence which comprises *orf1* (angMIII)*, *orf2* (angMII)*, and *orf3* (angMI)* from the angolamycin gene cluster.

Figure 10: The amino acid sequence which corresponds to *orf2 (angA1)*.

Figure 11: The amino acid sequence which corresponds to *orf3 (angAII)*.

25

Figure 12: The amino acid sequence which corresponds to *orf4*.

Figure 13: The amino acid sequence which corresponds to *orf14*.

30

Figure 14: The amino acid sequence which corresponds to *orf15 (angB)*.

Figure 15: The amino acid sequence which corresponds to *orf1* (angMIII)*.

Figure 16: The amino acid sequence which corresponds to *orf2* (angMII)*.

35

Figure 17: The amino acid sequence which corresponds to *orf3** (*angMT*).

General Methods

5 *Escherichia coli* XL1-Blue MR (Stratagene), *E. coli* DH10B (GibcoBRL) and *E. coli* ET12567 were grown in 2xTY medium as described by Sambrook *et al.*, (1989). Vector pUC18, pUC19 and Litmus 28 were obtained from New England Biolabs. *E. coli* transformants were selected with 100 µg/ml ampicillin. Conditions used for growing the *Saccharopolyspora erythraea* NRRL 2338-red variant strain were as described previously 10 (Gaisser *et al.*, 1997, Gaisser *et al.*, 1998). Expression vectors in *S. erythraea* were derived from plasmid pSG142 (Gaisser *et al.*, 2000). Plasmid-containing *S. erythraea* were selected with 25-40 µg/ml thiostrepton or 50 µg/ml apramycin. To investigate the production of antibiotics, *S. erythraea* strains were grown in sucrose-succinate medium (Caffrey *et al.*, 15 1992) as described previously (Gaisser *et al.*, 1997) and the cells were harvested by centrifugation. Chromosomal DNA of *Streptomyces rochei* ATCC21250 was isolated using standard procedures (Kieser *et al.*, 2000). Feedings of 3-*O*-mycarosyl erythronolide B or tylactone were carried out at concentrations between 25 to 50 mg /l.

DNA manipulation and sequencing

20 DNA manipulations, PCR and electroporation procedures were carried out as described in Sambrook *et al.*, (1989). Protoplast formation and transformation procedures of *S. erythraea* were as described previously (Gaisser *et al.*, 1997). Southern hybridizations were carried out with probes labelled with digoxigenin using the DIG DNA labelling kit 25 (Boehringer Mannheim). DNA sequencing was performed as described previously (Gaisser *et al.*, 1997), using automated DNA sequencing on double stranded DNA templates with an ABI Prism 3700 DNA Analyzer. Sequence data were analysed using standard programs.

Extraction and mass spectrometry

30 1 ml of each fermentation broth was harvested and the pH was adjusted to pH 9. For extractions an equal volume of ethyl acetate, methanol or acetonitrile was added, mixed for at least 30 min and centrifuged. For extractions with ethyl acetate, the organic layer was evaporated to dryness and then re-dissolved in 0.5 ml methanol. For methanol and acetonitrile extractions, supernatant was collected after centrifugation and used for analysis. High resolution spectra were obtained on a Bruker BioApex II FT-ICR (Bruker, Bremen, FRG).

Analysis of culture broths

An aliquot of whole broth (1 ml) was shaken with CH₃CN (1 ml) for 30 minutes. The mixture was clarified by centrifugation and the supernatant analysed by LCMS. The HPLC system comprised an Agilent HP1100 equipped with a Luna 5 µm C18 BDS 4.6 × 250 mm column

5 (Phenomenex, Macclesfield, UK) heated to 40°C. The gradient elution was from 25% mobile phase B to 75% mobile phase B over 19 minutes at a flow rate of 1 ml/min. Mobile phase A was 10% acetonitrile: 90% water, containing 10 mM ammonium acetate and 0.15% formic acid, mobile phase B was 90% acetonitrile: 10% water, containing 10 mM ammonium acetate and 0.15% formic acid. The HPLC system described was coupled to a Bruker Daltonics

10 Esquire3000 electrospray mass spectrometer operating in positive ion mode.

Extraction and purification protocol:

For NMR analysis of 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A the fermentation broth was clarified by centrifugation to provide supernatant and cells. The

15 supernatant was applied to a column (16 × 15 cm) of Diaion® HP20 resin (Supelco), washed with 10% Me₂CO/H₂O (2 × 2 l) and then eluted with Me₂CO (3.5 l). The cells were mixed to homogeneity with an equal volume of Me₂CO/MeOH (1:1). After at least 30 minutes the slurry was clarified by centrifugation and the supernatant decanted. The pelleted cells were similarly extracted once more with Me₂CO/MeOH (1:1). The cell extracts were combined
20 with the Me₂CO from the HP20 column and the solvent was removed *in vacuo* to give an aqueous concentrate. The aqueous was extracted with EtOAc (3 ×) and the solvent removed *in vacuo* to give a crude extract. The residue was dissolved in CH₃CN/MeOH and purified by repeated rounds of reverse phase (C18) high performance liquid chromatography using a Gilson HPLC, eluting a Phenomenex 21.2 × 250 mm Luna 5 µm C18 BDS column at 21
25 ml/min. Elution with a linear gradient of 32.5% B to 63% B was used to concentrate the macrolides followed by isocratic elution with 30% B to resolve the individual erythromycins. Mobile phase A was 20 mM ammonium acetate and mobile phase B was acetonitrile.
High resolution mass spectra were acquired on a Bruker BioApex II FTICR (Bruker, Bremen,
Germany).

30

For NMR analysis of 5-O-angulosaminyl tylactone bioconversion experiments were performed as previously described with four 2 l flasks containing each 400 ml of SSDM medium inoculated with 5% of pre-cultures. Feedings with tylactone were carried out at 50 mg/l. The culture was centrifuged and the pH of the supernatant was adjusted to about pH 9
35 followed by extractions with three equal volumes of ethyl acetate. The cell pellet was

extracted twice with equal volumes of a mixture of acetone-methanol (50:50, vol/vol). The extracts were combined and concentrated *in vacuo*. The resulting aqueous fraction was extracted three times with ethyl acetate and the extracts were combined and evaporated until dryness. This semi purified extract was dissolved in methanol and purified by preparative

- 5 HPLC on a Gilson 315 system using a 21 mm × 250 mm Prodigy ODS3 column (Phenomenex, Macclesfield, UK). The mobile phase was pumped at a flow rate of 21 ml/min as a binary system consisting of 30% CH₃CN, 70% H₂O increasing linearly to 70% CH₃CN over 20 min.

10 *Sequence Information*

Table I – Sequence information for the angulosamine biosynthetic genes included in the gene cassettes

Gene (named according to tyl equivalent)	Bases in Figure	Corresponding polypeptide Figure number
<i>orf2 (angA1)</i>	14847-15731c from Figure 8	Figure 10 NDP-hexose synthase
<i>orf3 (angAII)</i>	13779-14774c from Figure 8	Figure 11 NDP-hexose 4,6-dehydratase
<i>orf4</i> (N-part) (C-part)	11306-13666c from Figure 8	Figure 12 typeII thioesterase NDP-hexose 2,3-dehydratase
<i>orf14</i>	1162-2160c from Figure 7	Figure 13 NDP-hexose 4-ketoreductase
<i>orf15 (angB)</i>	33-1151c from Figure 7	Figure 14 NDP-hexoseaminotransferase
<i>orf1* (angMIII)</i>	59800-61140 from Figure 9	Figure 15 Hypothetical NDP hexose 3,4 isomerase
<i>orf2* (angMII)</i>	61159-62430 from Figure 9	Figure 16 angulosaminyl glycosyl transferase
<i>orf3* (angMI)</i>	62452-63171 from Figure 9	Figure 17 N,N-dimethyl transferase

Note : c indicates that the gene is encoded by the complement DNA strand
potential functions of the predicted polypeptides (SEQ ID No.7 to 34) were obtained
from the NCBI database using a BLAST search.

Example 1: Bioconversion of 3-O-mycarosyl erythronolide B to 5-O-dedesosaminyl-5-O-mycaminosyl erythromycins using gene cassette pSG144tylAItylAIItylMIIItylBtylIatyItylMIIeryCIII.

5

Isolation of pSG143

Plasmid pSG142 (Gaisser *et al.*, 2000) was digested with *Xba*I and a fill-in reaction was performed using standard protocols. The DNA was re-ligated and used to transform *E. coli* DH10B. Construct pSG143 was isolated and the removal of the *Xba*I site was confirmed by sequence analysis.

10

Isolation of pUC18eryBVcas

The gene *eryBV* was amplified by PCR using the primers casOleG21 (WO01/79520) and 7966 5'-GGGAATTCAGATCTGGTCTAGAGGTCAGCCGGCGTGGCGGCGCGTG AGTTCCAGTCGGGACGATCT -3' and pSG142 (Gaisser *et al.*, 2000) as template. The PCR fragment was cloned using standard procedures and plasmid pUC18eryBVcas was isolated with an *Nde*I site overlapping the start codon of *eryBV* and *Xba*I and *Bgl*II sites (underlined) following the stop codon. The construct was verified by sequence analysis.

20

Isolation of vector pSGLit1

The isolation of this vector is described in PCT/GB03/003230.

Isolation of pSGLit1eryCIII

Plasmid pSGCIII (WO01/79520) was digested with *Nde*I/*Bgl*II and the insert fragment was isolated and ligated with the *Nde*I/*Bgl*II treated vector fragment of pSGLit1. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit1eryCIII was isolated using standard procedures. The construct was confirmed using restriction digests and sequence analysis. This cloning strategy allows the introduction of a *his*-tag C-terminal of EryCIII.

30

Isolation of pSGLit1tylMII

Plasmid pSGTYLM2 (WO01/7952) was digested with *Nde*I/*Bgl*II and the insert fragment was isolated and ligated with the *Nde*I/*Bgl*II treated vector fragment of pSGLit1. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit1tylMII was isolated using standard procedures. The construct was confirmed using restriction digests and sequence analysis. This cloning strategy allows the introduction of a *his*-tag C-terminal of TylMII.

35

Isolation of pSG144

Plasmid pSGLit1 was isolated and digested with *NdeI/BglII* and an approximately 1.3 kb insert was isolated. Plasmid pSG143 was digested with *NdeI/BglII*, the vector band was isolated and ligated with the approximately 1.3 kb band from pSGLit1 followed by transformation of *E. coli* DH10B. Plasmid pSG144 (F₊) was isolated and the construct was verified by DNA sequence analysis. This vector allows the assembly of gene cassettes directly in an expression vector (Figure 2) without prior assembly in pUC-derived vectors (WO 01/79520) in analogy to PCT/GB03/003230 using vector pSG144 instead of pSGset1.

Plasmid pSG144 differs from pSG142 in that the *XbaI* site between the thiostrepton resistance gene and the eryRHS has been deleted and the *his-* tag at the end of *eryBV* has been removed from pSG142 and replaced in pSG144 with an *XbaI* site at the end of *eryBV*. This is to facilitate direct cloning of genes to replace *eryBV* and then build up the cassette.

15 *Isolation of pSG144eryCIII*

EryCIII was amplified by PCR reaction using standard protocols, with primers casOleG21 (WO 01/79520) and caseryCIII2 (WO 01/79520) and plasmid pSGCIII (Gaisser *et al.*, 2000) as template. The approximately 1.3 kb PCR product was isolated and cloned into pUC18 using standard techniques. Plasmid pUCCIIIcass was isolated and the sequence was verified. The insert fragment of plasmid pUCCIIIcass was isolated after *NdeI/XbaI* digestion and ligated with the *NdeI/XbaI* digested vector fragment of pSG144. After the transformation of *E. coli* DH10B plasmid pSG144eryCIII was isolated using standard techniques.

25 *Isolation of pUC19tylAI*

Primers BIOSG34 5'-
GGGCATATGAACGACCGTCCCCGCCGCCATGAAGGG- 3' and 5'-
CCCCTCTAGAGGTCACTGTGCCCGGCTGTCGGCGGCCCGCGCATGG-3' were used with genomic DNA of *Streptomyces fradiae* as template to amplify *tylAI*. The amplified product was cloned using standard protocols and plasmid pUC19tylAI was isolated. The insert was verified by DNA sequence analysis. Differences to the published sequence are shown in Figure 3.

Isolation of pSGLit2

Plasmid Litmus 28 was digested with *SpeI/XbaI* and the vector fragment was isolated. 35 Plasmid pSGLit1 (*dam*) was digested with *XbaI* and the insert band was isolated and ligated

with the *SpeI/XbaI* digested vector fragment of Litmus 28 followed by the transformation of *E. coli* DH10B using standard techniques. Plasmid pSGLit2 was isolated and the construct was verified by restriction digest and sequence analysis. This plasmid can be used to add a 5' region containing an *XbaI* site sensitive to Dam methylation and a Shine Dalgarno region thus converting genes which were originally cloned with an *NdeI* site overlapping the start codon and an *XbaI* site 3' of the stop codon for the assembly of gene cassettes. This conversion includes the transformation of the ligations into *E. coli* ET12567 followed by the isolation of *dam*⁻ DNA and *XbaI* digests. Examples for this strategy are outlined below.

10 *Isolation of pSGLit2tylAI*

Plasmid pSGLit2 and pUC19*tylAI* were digested with *NdeI* / *XbaI* and the insert band of pUC19*tylAI* and the vector band of pSGLit2 were isolated, ligated and used to transform *E. coli* ET12567. Plasmid pSGLit2*tylAI* (*dam*⁻) was isolated.

15 *Isolation of pUC19tylAII*

Primers 5' –

CCCTCTAGAGGTCATGCGCGCTCCAGTTCCCTGCCGCCGGGGACCGCTTG- 3'

and 5' –

GGGTCTAGATCGATTAATTAAGGAGGGACATTCA

20 TGCGGGCTTCATCGGCTCGCACTTCA- 3' and genomic DNA of *Streptomyces fradiae* as template were used for a PCR reaction applying standard protocols to amplify *tylAII*. The approximately 1 kb sized DNA fragment was isolated and cloned into *SmaI*-cut pUC19 using standard techniques. The DNA sequencing of this construct revealed that 12 nucleotides at the 5' end had been removed possibly by an exonuclease activity present in the PCR reaction.

25 The comparison of the amino acid sequence of the cloned fragment compared to the published sequence is shown in Figure 4.

Isolation of pSGLit2tylAII

To add the missing 5'-nucleotides, pSGLit2 was digested with *PacI/XbaI* and the 30 vector fragment was isolated and ligated with the *PacI/XbaI* digested insert fragment of pUC19*tylAII*. The ligated DNA was used to transform *E. coli* ET12567 and plasmid pSGLit2*tylAII* (*dam*⁻) was isolated.

Isolation of plasmid pUC19eryCVI

The *eryCVI* gene was amplified by PCR using primer BIOSG28 5'-

GGGCATATGTACGAGGGCGGGTTCGCCGAGCTTACGACC-3' and BIOSG29 5'-

GGGGTCTAGAGGTCATCCGCGCACACCGACGAACAACCCG-3' and plasmid

- 5 pNCO62 (Gaisser *et al.*, 1997) as a template. The PCR product was cloned into *Sma*I digested pUC19 using standard techniques and plasmid pUC19*eryCVI* was isolated and verified by sequence analysis.

Isolation of plasmid pSGLit2eryCVI

- 10 Plasmid pUC19*eryCVI* was digested with *Nde*I/*Xba*I and ligated with the *Nde*I/*Xba*I digested vector fragment of pSGLit2 followed by transformation of *E. coli* ET12567. Plasmid pSGLit2*eryCVI* (*dam*⁻) was isolated.

Isolation of plasmid pSG144tylAI

- 15 Plasmid pSG144 and pUC19*tylAI* were digested with *Nde*I/*Xba*I and the insert band of pUC19*tylAI* and the vector band of pSG144 were isolated, ligated and used to transform *E. coli* DH10B. Plasmid pSG144*tylAI* was isolated using standard protocols.

Isolation of plasmid pSG144tylAItylAII

- 20 Plasmid pSGLit2*tylAII* (*dam*⁻) was digested with *Xba*I and ligated with *Xba*I digested plasmid pSG144*tylAI*. The ligation was used to transform *E. coli* DH10B and plasmid pSG144*tylAItylAII* was isolated and verified using standard protocols.

*Isolation of plasmid pSGLit2*tylMIII**

- 25 Plasmid pUC18*tylM3* (Isolation described in WO01/79520) was digested with *Nde*I/*Xba*I and the insert band and the vector band of *Nde*I/*Xba*I digested pSGLit2 were isolated, ligated and used to transform *E. coli* ET12567. Plasmid pSGLit2*tylMIII* (*dam*⁻) was isolated using standard protocols. The construct was verified using restriction digests and sequence analysis.

30 *Isolation of plasmid pSG144*tylAItylAIItylMIII**

- Plasmid pSGLit2*tylMIII* (*dam*⁻) was digested with *Xba*I and the insert band was ligated with *Xba*I digested plasmid pSG144*tylAItylAII*. The ligation was used to transform *E. coli* DH10B and plasmid pSG144*tylAItylAIItylMIII* no36 was isolated using standard protocols. The construct was verified using restriction digests and sequence analysis.

Isolation of plasmid pSGLit2tylB

Plasmid pUC18tylB (Isolation described in WO01/79520) was digested with *PacI/XbaI* and the insert band and the vector band of *PacI/XbaI* digested pSGLit2 were isolated, ligated and used to transform *E. coli* ET12567. Plasmid pSGLit2tylB no1 (*dam*⁻) was isolated using standard protocols.

Isolation of plasmid pSG144tylAIItylAIItylMIIItyIIB

Plasmid pSGLit2tylB (*dam*⁻) was digested with *XbaI* and the insert band was ligated with *XbaI* digested plasmid pSG144tylAIItylAIItylMIII. The ligation was used to transform *E. coli* DH10B and plasmid pSG144tylAIItylAIItylMIIItyIIB no5 was isolated using standard protocols and verified by restriction digests and sequence analysis.

Isolation of plasmid pUC18tylla

Primers BIOSG 88 5'-GGGCATATGGCGCGAGCACTACGACGGAGGGAAATGT-3' and BIOSG 89 5'-GGGTCTAGAGGTACGGGTGGCTCCTGCCGCCCTCAG-3' were used to amplify *tylla* using a plasmid carrying the *tyl* region (accession number u08223.em_pro2) comprising ORF1 (cytochrome P450) to the end of ORF2 (TylB) as a template. Plasmid pUCtylla no1 was isolated using standard procedures and the construct was verified using sequence analysis.

Isolation of plasmid pSGLit2tylla

Plasmid pUCtylla no1 was digested with *NdeI/XbaI* and the insert band and the vector band of *NdeI/XbaI* digested pSGLit2 were isolated, ligated and used to transform *E. coli* ET12567. Plasmid pSGLit2tylla no 54 (*dam*⁻) was isolated using standard protocols. The construct was verified using sequence analysis.

Isolation of plasmid pSG144tylAIItylAIItylMIIItyIIBtylla

Plasmid pSGLit2tylla (*dam*⁻) was digested with *XbaI* and the insert band was ligated with *XbaI* digested plasmid pSG144tylAIItylAIItylMIIItyIIB. The ligation was used to transform *E. coli* DH10B and plasmid pSG144tylAIItylMIIItyIIBtylla no3 was isolated using standard protocols and verified by restriction digests and sequence analysis.

Isolation of plasmid pSGLitItylMeryCIII

Plasmid pUCtylM (Isolation described in WO01/79520) was *PacI* digested and the insert was ligated with the *PacI* digested vector fragment of pSGLit1*eryCIII* using standard procedures.

Plasmid pSGLit1tylM_IeryC_{III} no20 was isolated and the orientation was confirmed by restriction digests and sequence analysis.

Isolation of gene cassette pSG144tylAIItylAItylMIIItylBtylIatyIeryCIII

5 Plasmid pSGLit1tylM_IeryC_{III} no20 was digested with *Xba*I/*Bgl*II and the insert band was isolated and ligated with the *Xba*I/*Bgl*II digested vector fragment of plasmid pSG144tylAIItylAItylMIIItylBtylIa no3. Plasmid pSG144tylAIItylAItylMIIItylBtylIatyIeryC_{III} was isolated using standard procedures and the construct was confirmed using restriction digests and sequence analysis. Plasmid 10 preparations were used to transform *S. erythraea* mutant strains with standard procedures.

Isolation of plasmid pSGKC1

To prevent the conversion of the substrate 3-*O*-mycarosyl erythronolide B to 3,5-di-*O*-mycarosyl erythronolide B a further chromosomal mutation was introduced into *S. erythraea* SGQ2 (Isolation described in WO 01/79520) to prevent the biosynthesis of L-mycarose in the strain background. Plasmid pSGKC1 was isolated by cloning the approximately 0.7 kb DNA fragment of the *eryBV1* gene by using PCR amplification with cosmid2 or plasmid pGG1 (WO01/79520) as a template and with the primers 646 5'-
15 CATCGTCAAGGAGTTGACGGT- 3' and 874 5'-GCCAGCTGGCGACGTCCATC-
20 3' using standard protocols. Cosmid 2 containing the right hand site of the *ery*-cluster was isolated from an existing cosmid library (Gaisser *et al.*, 1997) by screening with *eryBV* as a probe using standard techniques. The amplified DNA fragment was isolated and cloned into *Eco*RV digested pKC1132 (Bierman *et al.*, 1992) using standard methods. The ligated DNA was used to transform *E. coli* DH10B and plasmid pSGKC1 was isolated using standard 25 molecular biological techniques. The construct was verified by DNA sequence analysis.

Isolation of S. erythraea Q42/1 (Biot-2166)

Plasmid pSGKC1 was used to transform *S. erythraea* SGQ2 using standard techniques followed by selection with apramycin. Thiostrepton/apramycin resistant transformant *S. erythraea* Q42/1 was isolated.

Bioconversion using S. erythraea Q42/1 pSG144tylAIItylAItylMIIItylBtylIatyIeryCIII

Bioconversion assays using 3-*O*-mycarosyl erythronolide B are carried out as described in General Methods. Improved levels of mycaminosyl erythromycin A are detected in 35 bioconversion assays using *S. erythraea*

Q42/1pSG144tylAItylAIItylMIIItylBtylIatylM_IeryC_{III} compared to bioconversion levels previously observed (WO01/79520).

Example 2: Isolation of mycaminosyl tylactone using gene cassette

5 **pSG144tylAItylAIItylMIIItylBtylIatylM_ItylM_{II}**

Isolation of plasmid pSGLit1tylM_ItylM_{II}

Plasmid pUCtylM_I (Isolation described in WO01/79520) was *PacI* digested and the insert was ligated with the *PacI* digested vector fragment of pSGLit1tylM_{II} using standard procedures.

10 Plasmid pSGLit1tylM_ItylM_{II} no16 was isolated and the construct was confirmed by restriction digests and sequence analysis.

Isolation of plasmid pSG144tylAItylAIItylMIIItylBtylIatylM_ItylM_{II}

Plasmid pSGLit1tylM_ItylM_{II} no16 was digested with *Xba*I/*Bgl*II and the insert band was

15 isolated and ligated with the *Xba*I/*Bgl*II digested vector fragment of plasmid pSG144tylAItylAIItylMIIItylBtylIa no3. Plasmid

pSG144tylAItylAIItylMIIItylBtylIatylM_ItylM_{II} was isolated using standard procedures and the construct was confirmed using restriction digests and sequence analysis. The plasmid was isolated and used for transformation of *S. erythraea* mutant strains using standard protocols.

20

Bioconversion using gene cassette pSG144tylAItylAIItylMIIItylBtylIatylM_ItylM_{II}

The conversion of fed tylactone to mycaminosyl tylactone was assessed in bioconversion assays using *S. erythraea* Q42/1pSG144tylAItylAIItylMIIItylBtylIatylM_ItylM_{II}.

25 Bioconversion assays were carried out using standard protocols (see Chemical Request sheet 81). The analysis of the culture showed the major ion to be 568.8 [M+H]⁺ consistent with the presence of mycaminosyl tylactone. Fragmentation of this ion gave a daughter ion of m/z 174, as expected for protonated mycaminose. No tylactone was detected during the analysis of the culture extracts, indicating that the bioconversion of the fed tylactone was complete.

Recently, a homologue of Tyl_{II} was identified in the biosynthetic pathway of dTDP-3-

30 acetamido-3,6-dideoxy-alpha-D-galactose in *Aneurinibacillus thermoerophilus* L420-91^{T*} (Pfoestl *et al.*, 2003) and the function was postulated as a novel type of isomerase capable of synthesizing dTDP-6-deoxy-D-xylohex-3-ulose from dTDP-6-deoxy-D-xylohex-4-ulose.

Example 3: Bioconversion of 3-O-mycarosyl erythronolide B to 5-O-dedesosaminyl-5-O-mycaminosyl erythromycins using gene cassette pSG1448/27/95/21/44/193/6eryCIII (pSG144angA1angAIIorf14angMIIIangBangMIeryCIII).

5 *Cloning of angMII by isolating plasmid Lit1/4*

The gene *angMII* was amplified by PCR using the primers BIOSG61 5'-
GGGCATATGAGCCCCGCACCCGCCACCGAGGACCC -3' and BIOSG62 5'-
GGTCTAGAGGTCAGTTCGCGGTGCGGTGGCGGGCAGGTCAC -3'. Cosmid5B2
containing a fragment of the angolamycin biosynthetic pathway was used as template. The 1.4
10 kb PCR fragment (PCR no1) was cloned using standard procedures and *EcoRV* digested
plasmid Litmus28. Plasmid Lit1/4 was isolated with an *NdeI* site overlapping the start codon
of *angMII* and an *XbaI* site following the stop codon. The construct was verified by sequence
analysis.

15 *Isolation of plasmid pSGLit21/4*

Plasmid Lit1/4 was digested with *NdeI/XbaI* and the about 1.4 kb fragment was isolated and
ligated to *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli*
ET12567 and plasmid pSGLit21/4 no7 (*dam*⁻) was isolated. This construct was digested with
XbaI and used for the construction of gene cassettes.

20

Cloning of angMII by isolating plasmid Lit2/8

The gene *angMII* was amplified by PCR using the primers BIOSG63 5'-
GGGCATATGCGTATCCTGCTGACGTCGTTCGCGCACAAACAC -3' and BIOSG64 5'-
GGTCTAGAGGTCAGGCGCGCGGTGCGCGCGGTGAGGCGTCG -3' and
cosmid5B2 containing a fragment of the angolamycin biosynthetic pathway was used as
template. The 1.3 kb PCR fragment (PCR no2) was cloned using standard procedures and
EcoRV digested plasmid Litmus28. Plasmid Lit2/8 was isolated with an *NdeI* site overlapping
the start codon of *angMII* and an *XbaI* site following the stop codon. The construct was
verified by sequence analysis.

30

Cloning of angMII by isolating plasmid pLitangMII(BglII)

The gene *angMII* was amplified by PCR using primers BIOSG63 5'-
GGGCATATGCGTATCCTGCTGACGTCGTTCGCGCACAAACAC -3' and BIOSG80 5'-
GGAGATCTGGCGCGCGGTGCGCGCGGTGAGGCGTCG -3' and cosmid5B2
containing a fragment of the angolamycin biosynthetic pathway as template. The 1.3 kb PCR

fragment was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid Lit_{angMII(BglII)}no8 was isolated with an *NdeI* site overlapping the start codon of *angMII* and a *BglII* site instead of a stop codon thus allowing the addition of a *his-tag*. The construct was verified by sequence analysis.

5

Isolation of plasmid pSGLit1angMII

Plasmid Lit_{angMII(BglII)} was digested with *NdeI/BglII* and ligated with the *NdeI/BglII* digested vector fragment of pSGLit1. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit1_{angMII (dam)} was isolated using standard procedures.

10

Cloning of angMII by isolating plasmid Lit3/6

The gene *angMII* was amplified by PCR using the primers BIOSG65 5'-
GGGCATATGAACCTCGAATACAGCGGCGACATCGCCCGGTTG -3' and BIOSG66 5'-
GGTCTAGAGGTCAGGCCTGGACGCCGACGAAGAGTCCGCGGTCTG -3' and

15

cosmid5B2 containing a fragment of the angolamycin biosynthetic pathway was used as template. The 0.75 kb PCR fragment (PCR no3) was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid Lit3/6 was isolated with an *NdeI* site overlapping the start codon of *angMII* and an *XbaI* site following the stop codon. The construct was verified by sequence analysis.

20

Isolation of plasmid pSGLit23/6 no8

Plasmid Lit3/6 was digested with *NdeI/XbaI* and the about 0.8 kb fragment was isolated and ligated to *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit23/6 no8 (*dam*) was isolated. This construct was digested with *XbaI* and the isolated about 1 kb fragment was used for the assembly of gene cassettes.

Cloning of angB by isolating plasmid Lit4/19

The gene *angB* was amplified by PCR using the primers BIOSG67 5'-
GGGCATATGACTACCTACGTCTGGGACTACCTGGCGG -3' and BIOSG68 5'-
30 GGTCTAGAGGTCAGAGCGTGGCCAGTACCTCGTGCAGGGC -3' and cosmid4H2 containing a fragment of the angolamycin biosynthetic pathway was used as template. The 1.2 kb PCR fragment (PCR no4) was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid Lit4/19 was isolated with an *NdeI* site overlapping the start codon of *angB* and an *XbaI* site following the stop codon. The construct was verified by sequence analysis.

Isolation of plasmid pSGLit24/19

Plasmid Lit4/19 was digested with *NdeI/XbaI* and the 1.2 kb fragment was isolated and ligated into *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli*

- 5 ET12567 and plasmid pSGLit24/19 no24 (*dam*) was isolated. This construct was digested with *XbaI* and the isolated 1.2 kb fragment was used for the assembly of gene cassettes.

Cloning of orf14 by isolating plasmid Lit5/2

The gene *orf14* was amplified by PCR using the primers BIOSG69 5'-

- 10 GGGCATATGGTGAACGATCCGATGCCCGCGCGCAGTGGCAG-3' and BIOSG70 5'-
GGTCTAGAGGTCAACCTCCAGAGTGTTCGATGGGGTGGTGGG-3' and cosmid4H2 containing a fragment of the angolamycin biosynthetic pathway was used as template. The 1.0 kb PCR fragment (PCR no5) was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid Lit5/2 was isolated with an *NdeI* site overlapping the start codon
15 of *ORF14* and an *XbaI* site following the stop codon. The construct was verified by sequence analysis.

Isolation of plasmid pSGLit25/2 no24

Plasmid Lit5/2 was digested with *NdeI/XbaI* and the approximately 1 kb fragment was

- 20 isolated and ligated to *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit25/2 no24 (*dam*) was isolated. This construct was digested with *XbaI*, the about 1 kb fragment isolated and used for the assembly of gene cassettes.

25 *Isolation of plasmid pSGLit27/9 no15*

Plasmid Lit7/9 was digested with *NdeI/XbaI* and the approximately 1 kb fragment was isolated and ligated to *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit27/9 no15 (*dam*) was isolated. This construct was digested with *XbaI* and the isolated 1 kb fragment was used for the assembly of gene cassettes.

- 30

Cloning of angAI (orf2) by isolating plasmid Lit8/2

The gene *angAI* was amplified by PCR using the primers BIOSG73 5'-

GGGCATATGAAGGGCATCATCCTGGCGGGCGGCAGCGGC-3' and BIOSG74 5'-

- 35 GGTCTAGAGGTATGCGGCCGGTCCGGACATGAGGGTCTCCGCCAC-3' and

cosmid4H2 containing a fragment of the angolamycin biosynthetic pathway was used as template. The around 1.0 kb PCR fragment (PCR no8) was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid Lit8/2 was isolated with an *NdeI* site overlapping the start codon of *angAII* and an *XbaI* site following the stop codon. The construct
5 was verified by sequence analysis.

Cloning of angAII (orf3) by isolating plasmid Lit7/9

The gene *angAII* was amplified by PCR using the primers BIOSG71 5'-
GGGCATATCGGGCTGCTGGTCACCGGAGGTGCGGGC-3' and BIOSG72 5'-

10 GGTCTAGAGGTCAGTGGTGCGCCGGGCCTGCG-3' and cosmid4H2 containing a fragment of the angolamycin biosynthetic pathway was used as template. The 1.0 kb PCR fragment was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid Lit7/9 was isolated with an *NdeI* site overlapping the start codon of *angAII* and an *XbaI* site following the stop codon. The construct was verified by sequence analysis.
15

Isolation of plasmid pSGLit28/2 no18 (pSGLit2angAII)

Plasmid Lit8/2 was digested with *NdeI/XbaI* and the 1 kb fragment was isolated and ligated to *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit28/2 no18 (*dam*) was isolated.

20 *Isolation of plasmid pSG1448/2 (pSG144angAII)*
Plasmid Lit8/2 was digested with *NdeI/XbaI* and the approximately 1 kb fragment was isolated and ligated with *NdeI/XbaI* digested DNA of pSG144. The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/2 (*dam*) (pSG144angAII) was isolated using
25 standard procedures. This construct was verified with restriction digests and sequence analysis.

Isolation of plasmid pSG1448/27/9 (pSG144angAIIangAII)

30 Plasmid pSGLit27/9 (isolated from *E. coli*: ET12567) was digested with *XbaI* and the 1 kb fragment was isolated and ligated with the *XbaI* digested vector fragment of pSG1448/2 (pSG144angAII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/9 (pSG144angAIIangAII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

35 *Isolation of plasmid pSG1448/27/91/4 (pSG144angAIIangAIIangMIII)*

Plasmid pSGLit2I/4 (isolated from *E. coli* ET12567) was digested with *Xba*I and the 1.4 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/9 (pSG144angAIIangAII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/9I/4 (pSG144angAIIangAIIangMII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

5 *Isolation of plasmid pSG1448/27/9I/44/19 (pSG144angAIIangAIIangMIIangB)*

Plasmid pSGLit24/19 (isolated from *E. coli* ET12567) was digested with *Xba*I and the about 1.2 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/9I/4 (pSG144angAIIangAIIangMII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/9I/44/19 (pSG144angAIIangAIIangMIIangB) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

10 15 *Isolation of plasmid pSG1448/27/9I/44/193/6 (pSG144angAIIangMIIangBangMT)*

Plasmid pSGLit23/6 (isolated from *E. coli* ET12567) was digested with *Xba*I and the about 0.8 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/9I/44/19 (pSG144angAIIangMIIangB). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/9I/44/193/6 (pSG144angAIIangMIIangBangMT) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

20 25 *Isolation of plasmid pSG1448/27/9I/44/193/6eryCIII*

(pSG144angAIIangMIIangBangMIIeryCIII)

Plasmid pSGLit1eryCIII (isolated from *E. coli* ET12567) was digested with *Xba*I/*Bgl*II and the about 1.2 kb fragment was isolated and ligated with the *Xba*I digested and partially *Bgl*II digested vector fragment of pSG1448/27/9I/44/193/6 (pSG144angAIIangMIIangBangMT). The *Bgl*II partial digest was necessary due to the presence of a *Bgl*II site in *angB*. The ligation was used to transform *E. coli* DH10B and

30 plasmid pSG1448/27/9I/44/193/6eryCIII no9 (pSG144angAIIangMIIangBangMIIeryCIII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis. EryCIII carries a *his*-tag fusion at the end.

*Bioconversion of 3-O-mycarosyl erythronolide B to 5-O-dedesosaminyl-5-O-myccaminosyl erythromycin A using S. erythraea Q42/1pSG1448/27/91/44/193/6eryCIII no9
(pSG144angAIIangMIIangBangMleryCIII)*

The *S. erythraea* strain Q42/1pSG1448/27/91/44/193/6eryCIII was grown and bioconversions with fed 3-O-mycarosyl erythronolide B were performed as described in the General Methods. The cultures were analysed and a small amount of a compound with m/z 750 was detected consistent with the presence of 5-O-dedesosaminyl-5-O-myccaminosyl erythromycin A.

10 *Isolation of plasmid pSG1448/27/95/2 (pSG144angAIIangAIIorf14)*

Plasmid pSGLit25/2 (isolated from *E. coli* ET12567) was digested with *Xba*I and the about 1 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/9 (pSG144angAIIangAII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/95/2 (pSG144angAIIangAIIorf14) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

15 *Isolation of plasmid pSG1448/27/95/21/4 (pSG144angAIIangAIIorf14angMIII)*

Plasmid pSGLit21/4 (isolated from *E. coli* ET12567) was digested with *Xba*I and the 1.4 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/95/2 (pSG144angAIIangAIIorf14). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/95/21/4 (pSG144angAIIangAIIorf14angMIII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

25 *Isolation of plasmid pSG1448/27/95/21/44/19 (pSG144angAIIangAIIorf14angMIIIangB)*

Plasmid pSGLit24/19 (isolated from *E. coli* ET12567) was digested with *Xba*I and the 1.2 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/95/21/4 (pSG144angAIIangAIIorf14angMIII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/95/21/44/19

30 *(pSG144angAIIangAIIorf14angMIIIangB)* was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

*Isolation of plasmid pSG1448/27/95/21/44/193/6eryCIII
(pSG144angAIIangAIIorf14angMIIIangBangMleryCIII)*

- Plasmid pSG1448/27/91/44/193/*6eryCIII* no9 was digested with *Bgl*II and the about 2 kb fragment was isolated and ligated with the *Bgl*II digested vector fragment of pSG1448/27/95/21/44/19 (pSG144~~angA~~*IangAIIorf14angMIIIangB*). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/95/21/44/193/*6eryCIII*
- 5 (pSG144~~angA~~*IangAIIorf14angMIIIangB**angCIII*) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis. EryCIII carries a *his*-tag fusion at the end. The construct was used to transform *S. erythraea* SGQ2 using standard procedures.
- 10 *Bioconversion of 3-O-mycarosyl erythronolide B to 5-O-dedesosaminyl-5-O-myccaminoxy erythromycin A*
The *S. erythraea* strain SGQ2pSG1448/27/95/21/44/193/*6eryCIII* was grown and bioconversions with fed 3-O-mycarosyl erythronolide B were performed as described in the General Methods. The cultures were analysed and improved amounts of a compound with m/z 15 750 was detected consistent with the presence of 5-O-dedesosaminyl-5-O-myccaminoxy erythromycin A. Similar results were obtained with the *S. erythraea* strain Q42/1 containing the gene cassette pSG1448/27/95/21/44/193/*6eryCIII*.
16 mg of the compound with m/z 750 was purified and the structure of 5-O-dedesosaminyl-5-O-myccaminoxy erythromycin A was confirmed by NMR analysis (See Table I and Figure 1).

20 *Table II: ¹H and ¹³C NMR data for 5-O-dedesosaminyl-5-O-myccaminoxy erythromycin A (BC156)*

Position	δ_{H}	Multiplicity	Coupling	δ_{C}
1				175.4
2	2.83	dq	9.6, 7.1	44.9
3	3.91	dd	9.7, 1.6	80.0
4	2.00	m		39.1
5	3.53	d	6.8	85.4
6				74.8
7	1.66	dd	14.8, 2.2	38.5
	1.82	dd	14.8, 11.4	
8	2.69	dqd	11.3, 7.0, 2.2	44.9
				221.6
9				
10	3.06	qd	6.9, 1.3	38.0
11	3.81	d	1.3	68.9
12				74.6
13	5.04	dd	11.0, 2.3	76.8 ^a
14	1.47	dqd	14.3, 11.0, 7.2	21.1
	1.91	ddq	14.3, 7.5, 2.2	
15	0.83	dd	7.4, 7.4	10.6
16	1.18	d	7.1	16.0
17	1.03	d	7.4	9.7
18	1.44	s		26.6

Position	δ_H	Multiplicity	Coupling	δ_C
19	1.16	d	7.0	18.3
20	1.14	d	7.0	12.0
21	1.12	s		16.2
1'	4.87	d	4.8	96.4
2'	1.55	dd	15.2, 4.8	34.9
	2.32	dd	15.2, 0.9	
3'				72.8
4'	3.01	d	9.3	77.8
5'	3.99	dq	9.3, 6.2	65.6
6'	1.27	d	6.2	18.5
7'	1.23	s		21.4
8'	3.29	s		49.4
1''	4.43	d	7.4	103.3
2''	3.56	dd	10.5, 7.3	71.3
3''	2.48	dd	10.3, 10.3	70.6
4''	3.09	dd	9.9, 9.0	70.2
5''	3.31	dq	9.0, 6.1	72.9
6''	1.29	d	6.1	18.1
7''	2.58	s		41.7

^a This carbon was assigned from the HMQC spectrum

Example 4: Isolation of mycaminosyl tylactone

Isolation of plasmid pSG1448/27/95/21/44/193/6tylMII

5 (*pSG144angAIIangAIIorf14angMIIIangB3/6tylMII*)

Plasmid pSG1448/27/91/44/193/6tylMII no9 was digested with *Bg*II and the about 2 kb fragment was isolated and ligated with the *Bg*II digested vector fragment of pSG1448/27/95/21/44/19 (*pSG144angAIIangAIIorf14angMIIIangB*). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/95/21/44/193/6tylMII

10 (*pSG144angAIIangAIIorf14angMIIIangBangMIItylMII*) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

TylMII carries a *his*-tag fusion at the end.

Bioconversion of tylactone to mycaminosyl tylactone

15 The *S. erythraea* strain Q42/1pSG1448/27/95/21/44/193/6tylMII is grown and bioconversions with fed tylactone is performed as described in the General Methods. The cultures are analysed and a compound with m/z 568 is detected consistent with the presence of mycaminosyl tylactone.

Example 5: Isolation of 5-O-dedesosaminyl-5-O-angulosaminyl erythromycins using gene cassette pSG1448/27/91/4spnO5/2p4/193/6tylMII by bioconversion of 3-O-mycarosyl erythronolide B.

5 *Isolation of plasmid conv no1*

For the multiple use of promoter sequences in *act*-controlled gene cassettes a 240 bp fragment was amplified by PCR using the primers BIOSG78 5'-

GGGCATATGTGTCCTCCTTAATTAAATCGATGCGTTCGTCC-3' and BIOSG79 5'-

GGAGATCTGGTCTAGATCGTGTCCCCCTCCGCCTCGTGGTCCCTCACGC -3' and

10 plasmid pSG142 (Gaisser *et al.*, 2000) as template. The 0.2 kb PCR fragment (PCR no5) was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid conv no1 was isolated. The construct was verified by sequence analysis.

Isolation of pSGLit3relig1

15 Plasmid conv no1 was digested with *NdeI/BgII* and the about 0.2 kb fragment was isolated and ligated with the *BamHI/NdeI* digested vector fragment of pSGLit2. The ligation was used to transform *E. coli* DH10B and plasmid pSGLit3relig1 was isolated using standard procedures. This construct was verified using restriction digests and sequence analysis.

20 *Isolation of plasmid pSGLit34/19*

Plasmid Lit4/19 was digested with *NdeI/XbaI* and the 1.2 kb fragment was isolated and ligated to *NdeI/XbaI* digested DNA of pSGLit3. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit34/19 no23 was isolated. This construct was digested with *XbaI* and the isolated 1.4 kb fragment was used for the assembly of gene cassettes.

25

Cloning of orf4 by isolating plasmid Lit6/4

The gene *orf4* was amplified by PCR using the primers BIOSG75 5'-

GGGCATATGAGCACCCCTCCGCACCACCGTTCCG-3' and BIOSG76 5'-

GGTCTAGAGGTCAGTACAGCGTGTGGGCACACGCCACCAAG-3' and cosmid4H2

30 containing a fragment of the angolamycin biosynthetic pathway was used as template. The 2.5 kb PCR fragment (PCR no6) was cloned using standard procedures and *EcoRV* digested plasmid Litmus28. Plasmid Lit6/4 was isolated with an *NdeI* site overlapping the start codon of *orf4* and an *XbaI* site following the stop codon. The construct was verified by sequence analysis.

Isolation of plasmid pSGLit26/4 no9

Plasmid Lit6/4 was digested with *NdeI/XbaI* and the DNA was isolated and ligated to *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit26/4 no9 was isolated. This construct was confirmed by restriction digests
5 and sequence analysis.

Cloning of spnO by isolating plasmid pUC19spnO

The gene *spnO* from the spinosyn biosynthetic gene cluster of *Saccharopolyspora spinosa* was amplified by PCR using the primers BIOSG41 5'-

10 GGGCATATGAGCAGTTCTGTCGAAGCTGAGGCAAGTG-3' and BIOSG42 5'-
GGTCTAGAGGTCATGCCCCAACGCCACAAGCTATGCAGG-3' and genomic DNA of *S. spinosa* as template. The about 1.5 kb PCR fragment was cloned using standard procedures and *SmaI* digested plasmid pUC19. Plasmid pUC19spnO no2 was isolated with an
15 *NdeI* site overlapping the start codon of *spnO* and an *XbaI* site following the stop codon. The construct was verified by sequence analysis.

Isolation of plasmid pSGLit2spnO no4

Plasmid pUC19spnO was digested with *NdeI/XbaI* and the 1.5 kb fragment was isolated and ligated to *NdeI/XbaI* digested DNA of pSGLit2. The ligation was used to transform *E. coli* ET12567 and plasmid pSGLit2spnO no 4 was isolated using standard procedures. This
20 construct was digested with *XbaI* and the isolated 1.5 kb fragment was used for the assembly of gene cassettes.

Isolation of plasmid pSG1448/27/91/4spnO (pSG144angAIIangAIIangMIIIspnO)

25 Plasmid pSGLit2spnO no4 (isolated from *E. coli* ET12567) was digested with *XbaI* and the 1.5 kb fragment was isolated and ligated with the *XbaI* digested vector fragment of pSG1448/27/91/4 (pSG144angAIIangAIIangMIII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnO (pSG144angAIIangMIIIspnO) was isolated using standard protocols. The construct was verified with restriction digests and sequence
30 analysis.

Isolation of plasmid pSG1448/27/91/4spnO5/2 (pSG144angAIIangMIIIspnOangorf14)

Plasmid pSGLit25/2 no24 (isolated from *E. coli* ET12567) was digested with *XbaI* and the 1 kb fragment was isolated and ligated with the *XbaI* digested vector fragment of
35 pSG1448/27/91/4spnO (pSG144angAIIangMIIIspnO). The ligation was used to

transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnO5/2 (pSG144angAIIangAIIangMIIIspnOangorf14) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

- 5 *Isolation of plasmid pSG1448/27/91/4spnO5/2p4/19*
(pSG144angAIIangAIIangMIIIspnOangorf14pangB)
Plasmid pSGLit34/19 no23 (isolated from *E. coli* ET12567) was digested with *Xba*I and the about 1.4 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/91/4spnO5/2 (pSG144angAIIangAIIangMIIIspnOangorf14). The ligation was
10 used to transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnO5/2p4/19 (pSG144angAIIangAIIangMIIIspnOangorf14pangB) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis. ‘p’ indicates the presence of the promoter region in front of *angB* to emphasize the presence of multiple promoter sites in the construct.
15 *Isolation of plasmid pSG1448/27/91/4spnO5/2p4/193/6eryCIII*
(pSG144angAIIangAIIangMIIIspnOorf14pangBangMeryCIII)
Plasmid pSG1448/27/91/44/193/6eryCIII no9 was digested with *Bgl*II and the about 2 kb fragment was isolated and ligated with the *Bgl*II digested vector fragment of
20 pSG1448/27/91/4spnO5/2p4/19 (pSG144angAIIangAIIangMIIIspnOorf14pangB). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnO5/2p4/193/6eryCIII (pSG144angAIIangAIIangMIIIspnOorf14pangBangMeryCIII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis. EryCIII
25 carries a *his*-tag fusion at the end. ‘p’ indicates the presence of the promoter region in front of *angB* to emphasize the presence of multiple promoter sites in the construct. The plasmid construct was used to transform mutant strains of *S. erythraea* using standard procedures.

30 *Bioconversion of 3-O-mycarosyl erythronolide B to 5-O-dedesosaminyl-5-O-angulosaminyl erythromycins*
Strain *S. erythraea* Q42/1pSG1448/27/91/4spnO5/2p4/193/6eryCIII was grown and bioconversions with fed 3-O-mycarosyl erythronolide B were performed as described in the General Methods. The cultures were analysed and peaks with m/z 704, m/z 718 and m/z 734 consistent with the presence of angulosaminyl erythromycin D, B and A, respectively, were observed.

Example 6: Production of 5-O-angulosaminyl tylactone

Isolation of plasmid pSG1448/27/91/4spnO5/2p4/193/6tylMII

(pSG144angAIIangAIIangMIIispnOorf14pangBangMItylMII)

- 5 Plasmid pSG1448/27/91/44/193/6tylMII no9 was digested with *Bgl*II and the about 2 kb fragment was isolated and ligated with the *Bgl*II digested vector fragment of pSG1448/27/91/4spnO5/2p4/19 (pSG144angAIIangAIIangMIIispnOorf14pangB). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnO5/2p4/193/6tylMII
- 10 (pSG144angAIIangAIIangMIIispnOorf14pangBangMItylMII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis. TylMII carries a *his*-tag fusion at the end. The plasmid was used to transform mutant strains of *S. erythraea* applying standard protocols. ‘p’ indicates the presence of the promoter region in front of *angB* to emphasize the presence of multiple promoter sites in the construct.

15

Isolation of S. erythraea 18A1(BIOT-2634)

- To introduce a deletion comprising the PKS and majority of post PKS genes in *S. erythraea* a region of the left hand side of the *ery*- cluster (LHS) containing a portion of *eryCI*, the complete *ermE* gene and a fragment of the *eryBI* gene were cloned together with a region of the right hand side of the *ery*- cluster (RHS) containing a portion of the *eryBVII* gene, the complete *eryK* gene and a fragment of DNA adjacent to *eryK*. This construct should enable homologous recombination into the genome in both LHS and RHS regions resulting in the isolation of a strain containing a deletion between these two regions of DNA. The LHS fragment (2201 bp) was PCR amplified using *S. erythraea* chromosomal DNA as template and primers BI~~del~~Nde (5'-CCCATATGACCGGAGTCGAGGTACCGGGCTTG-3') and BI~~del~~Spe (5'-GATACTAGTCCGCCACGCACGTCGCTGAGCC-3'). Primer BI~~del~~Nde contains an *Nde*I restriction site (underlined) and primer BI~~del~~Spe contains a *Spe*I restriction site used for subsequent cloning steps. The PCR product was cloned into the *Sma*I restriction site of pUC19, and plasmid pLSB177 was isolated using standard procedures. The construct was confirmed by sequence analysis. Similarly, RHS (2158 bp) was amplified by PCR using *S. erythraea* chromosomal DNA as template and primers BVII~~del~~Spe (5'-TGCACTAGTGGCCGGCGCTCGACGTCATCGTCGACAT-3') and BVII~~del~~Eco (5'-TCGATATCGTGTCCCTGCGGTTCACCTGCAACGCTG-3'). Primer BVII~~del~~Spe contains a *Spe*I restriction site and primer BVII~~del~~Eco contains an *Eco*RV restriction site. The PCR product was cloned into the *Sma*I restriction site of pUC19 in the orientation with *Spe*I

positioned adjacent to *Kpn*I and *Eco*RV positioned adjacent to *Xba*I. The plasmid pLSB178 was isolated and confirmed using sequence analysis. Plasmid pLSB177 was digested with *Nde*I and *Spe*I, the ~2.2kb fragment was isolated and similarly plasmid pLSB178 was digested with *Nde*I and *Spe*I and the ~4.6 kb fragment was isolated using standard methods.

5 Both fragments were ligated and plasmid pLSB188 containing LHS and RHS combined together at a *Spe*I site in pUC19 was isolated using standard protocols. An *Nde*I/*Xba*I fragment (~4.4 kbp) from pLSB188 was isolated and ligated with *Spe*I and *Nde*I treated pCJR24. The ligation was used to transform *E. coli* DH10B and plasmid pLSB189 was isolated using standard methods. Plasmid pLSB189 was used to transform *S. erythraea* P2338 and transformants were selected using thiostrepton. *S. erythraea* Del18 was isolated and inoculated into 6 ml TSB medium and grown for 2 days. A 5% inoculum was used to subculture this strain 3 times. 100 µl of the final culture were used to plate onto R2T20 agar followed by an incubation at 30°C to allow sporulation. Spores were harvested, filtered, diluted and plated onto R2T20 agar using standard procedures. Colonies were replica plated onto R2T20 plates with and without addition of thiostrepton. Colonies that could no longer grow on thiostrepton were selected and further grown in TSB medium. *S. erythraea* 18A1 was isolated and confirmed using PCR and Southern blot analysis. The strain was designated LB-1 /BIOT-2634. For further analysis, the production of erythromycin was assessed as described in General Methods and the lack of erythromycin production was confirmed. In bioconversion assays this strain did not further process fed erythronolide B and erythromycin D was hydroxylated at C12 to give erythromycin C as expected, indicating that EryK was still functional.

Bioconversion of tylactone to 5-O- angulosaminyl tylactone

25 Strain *S. erythraea* SGQ2pSG1448/27/91/4spnO5/2p4/193/6tylMII was grown and bioconversions with fed tylactone were performed as described in the General Methods. The cultures were extracted and analysed. A compound consistent with the presence of angulosaminyl tylactone was detected. 20 mg of this compound were purified and the structure was confirmed by NMR analysis. A compound consistent with the presence of angulosaminyl tylactone was also obtained when the gene cassette pSG1448/27/91/4spnO5/2p4/193/6tylMII was expressed in the *S. erythraea* strain Q42/1 or *S. erythraea* 18A1.

Table III: NMR data for 5-O- β D angulosaminyl Tylactone

#	δ_c	δ_H (mult., Hz)	COSY H-H	HMBC H-C
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1	174.4			
2	39.8	1.91 d (16.8)	2b	1, 3
3	66.9	2.46 dd(16.8, 10.5)	2a, 3	1
4	40.4	3.68 dd (10.5, 1.2)	2b	1
5	80.7	1.56 m	5, 18	3
6	38.7	3.76 d (10.3)	4	4, 7, 18, 19, 1'
7	33.6	2.68 m	7b	
8	45.0	1.45 m		
9	203.9	1.55 m	6	
10	118.3	2.70 m	21	
11	147.7	6.26 d (15.5)	11	12
12	147.7	7.27 d (15.5)	10	9, 12, 13, 22
13	133.5	5.60 d (10.4)	14, 22	11, 14, 22, 23
14	145.4	2.70 m	13, 15, 23	12, 13, 15, 23
15	38.3	4.68 td (9.7, 2.4)	14, 16b	1, 17
16	78.8	1.55 m	15, 16b, 17	15
17	24.7	1.82 ddd	16a, 17	18
18	9.6	0.91 t (7.2)	16	15, 16
19	9.7	0.91 d (7.2)	4	3, 4, 5
20	21.0	1.55 m	20	
21	11.8	0.83 t (7.2)	19	6, 19
22	17.1	1.15 d (6.8)	8	7, 9
23	13.0	1.76 s	13	11, 12, 13
1'	16.1	0.83 t (6.5)	14	13, 14, 15
1'	101.0	4.41 d (8.6)	2'	2'
2'	28.0	1.48 m	1', 2b', 3'	1', 3', 4'
3'	2.05 ddd (10.4, 3.9, 1.6)	2a', 3'	1', 3'	
4'	65.8	2.89 td (10.0, 3.9)	2a', 2b', 4'	4'
5'	70.5	3.16 dd (9.5, 9.0)	3', 5'	3', 5', 6'
6'	73.2	3.26 dq (9.6, 6.0)	4', 6'	
6'	17.7	1.3 d (6.0)	5'	

Isolation of plasmid pSG1448/27/91/4spnOp5/2

(*pSG144angAIIangAIIangMIIIspnOpangorfI4*)

Plasmid pSGLit35/2 (isolated from *E. coli* ET12567) was digested with *Xba*I and the insert

5 fragment was isolated and ligated with the *Xba*I digested vector fragment of

pSG1448/27/91/4spnO (*pSG144angAIIangAIIangMIIIspnO*). The ligation was used to

transform *E. coli* DH10B and plasmid *pSG1448/27/91/4spnOp5/2*

(pSG144angAIIangMIIispnOpangorf14) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

Isolation of plasmid pSG1448/27/91/4spnOp5/24/19

5 (pSG144angAIIangMIIispnOpangorf14angB)

Plasmid pSGLit24/19 (isolated from *E. coli* ET12567) was digested with *Xba*I and the insert fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/91/4spnOp5/2 (pSG144angAIIangMIIispnOpangorf14). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnOp5/24/19

10 (pSG144angAIIangMIIispnOpangorf14angB) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

Isolation of plasmid pSG1448/27/91/4spnOp5/24/193/6

(pSG144angAIIangMIIispnOpangorf14angBangMII)

15 Plasmid pSGLit23/6 (isolated from *E. coli* ET12567) was digested with *Xba*I and the insert fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/91/4spnOp5/24/19 (pSG144angAIIangMIIispnOpangorf14angB). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnOp5/24/193/6 (pSG144angAIIangMIIispnOpangorf14angBangMII) was isolated using standard

20 protocols. The construct was verified with restriction digests and sequence analysis.

Isolation of plasmid pSG1448/27/91/4spnOp5/24/193/6angMII

(pSG144angAIIangMIIispnOpangorf14angBangMIIangMII)

25 Plasmid pSGLit1angMII (isolated from *E. coli* ET12567) was digested with *Xba*I/*Bgl*II and the insert fragment was isolated and ligated with the *Xba*I and partial *Bgl*II digested vector fragment of pSG1448/27/91/4spnOp5/24/193/6 (pSG144angAIIangMIIispnOpangorf14angBangMII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/91/4spnOp5/24/193/6angMII (pSG144angAIIangMIIispnOpangorf14angBangMIIangMII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis. The plasmid was used to transform mutant strains of *S. erythraea* with standard procedures.

Biotransformation using S. erythraea Q42/1 pSG1448/27/91/4spnOp5/24/193/6angMII

(pSG144angAIIangMIIispnOpangorf14angBangMIIangMII)

Biotransformation experiments feeding tylactone are carried out as described in General Methods and the cultures are analysed. Angolosaminyl tylactone is detected.

Isolation of plasmid pSG1448/27/96/4 (pSG144angAIIangorf4)

- 5 Plasmid pSG1448/27/9 (pSG144angAIIangorf4) was digested with *Xba*I and treated with alkaline phosphatase using standard protocols. The vector fragment was used for ligations with *Xba*I treated plasmid pSGLit26/4 no9 followed by transformations of *E. coli* DH10B using standard protocols. Plasmid pSG1448/27/96/4 (pSG144angAIIangorf4) was isolated using standard procedures and the construct was confirmed by restriction digests and
10 sequence analysis.

Isolation of plasmid pSG1448/27/96/4p5/2 (pSG144angAIIangorf4pangorf14)

Plasmid pSGLit35/2 (isolated from *E. coli* ET12567) was digested with *Xba*I and the insert fragment was isolated and ligated with the *Xba*I digested vector fragment of

- 15 pSG1448/27/96/4 (pSG144angAIIangorf4). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/96/4p5/2 (pSG144angAIIangorf4pangorf14) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

20 *Isolation of plasmid pSG1448/27/96/4p5/21/4*

(pSG144angAIIangorf4pangorf14angMIII)

Plasmid pSGLit21/4 (isolated from *E. coli* ET12567) was digested with *Xba*I and the 1.4 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/96/4p5/2 (pSG144angAIIangorf4pangorf14). The ligation was used to

- 25 transform *E. coli* DH10B and plasmid pSG1448/27/96/4p5/21/4 (pSG144angAIIangorf4pangorf14angMIII) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

Isolation of plasmid pSG1448/27/96/4p5/21/44/19

30 (pSG144angAIIangorf4pangorf14angMIIIangB)

Plasmid pSGLit24/19 (isolated from *E. coli* ET12567) was digested with *Xba*I and the 1.4 kb fragment was isolated and ligated with the *Xba*I digested vector fragment of pSG1448/27/96/4p5/21/4 (pSG144angAIIangorf4pangorf14angMIII). The ligation was used to transform *E. coli* DH10B and plasmid pSG1448/27/96/4p5/21/44/19

(pSG144angAIIangAIIangorf4pangorf14angMIIangB) was isolated using standard protocols. The construct was verified with restriction digests and sequence analysis.

Isolation of plasmid pSG1448/27/96/4p5/21/44/193/6angMII

- 5 (pSG144angAIIangAIIangorf4pangorf14angMIIangBangMIIangMII)
Plasmid pSG1448/27/91/4spnOp5/24/193/6angMII was digested with *Bgl*II and the about 2.2 kb fragment was isolated and used to ligate with the *Bgl*II treated vector fragment of pSG1448/27/96/4p5/21/44/19. The ligation was used to transform *E. coli* DH10B using standard procedures and plasmid pSG1448/27/96/4p5/21/44/193/6angMII
10 (pSG144angAIIangAIIangorf4pangorf14angMIIangBangMIIangMII) was isolated. The construct was verified using restriction digests and sequence analysis. The plasmid was used to transform mutant strains of *S. erythraea* with standard protocols.

Bioconversion of tylactone with S. erythraea Q42/1 pSG1448/27/96/4p5/21/44/193/6angMII

- 15 (pSG144angAIIangAIIangorf4pangorf14angMIIangBangMIIangMII)
Biotransformation experiments feeding tylactone are carried out as described in General Methods and the cultures are analysed. Angolosaminyloxytylactone is detected.

Example 7: Cloning of *eryK* into the gene cassette pSG144

- 20 *Isolation of plasmid pUC19eryK*
To amplify *eryK* primers eryK1 5'-
GGTCTAGACTACGCCACTGCCTGGCGAGGAGCCC-3' and eryK2: 5'-
GGCATATGTTGCCGACGTGGAAACGACCTGCTGCG-5' were used and the PCR product was cloned as described for pUC19eryCVI. Plasmid pUC19eryK was isolated.
25

Isolation of plasmid pLSB111 (pCJR24eryK)

Plasmid pUC19eryK was digested with *Nde*I/*Xba*I and the insert band was ligated with *Nde*I/*Xba*I digested pCJR24. Plasmid pLSB111 (pCJR24eryK) was isolated and the construct was verified with restriction digests.

- 30 *Isolation of plasmid pLSB115*
Plasmid pLSB111 (pCJR24eryK) was digested with *Nde*I/*Xba*I and the insert fragment was isolated and ligated with the *Nde*I/*Xba*I digested vector fragment of plasmid pSGLit2 and plasmid pLSB115 was isolated using standard protocols. The plasmid was verified using restriction digestion and DNA sequence analysis.
35

Isolation of plasmid pSG1448/27/95/21/4eryK

Plasmid pLSB115 from *E. coli* ET12567 was digested with *Xba*I and the insert fragment was isolated and ligated with the *Xba*I treated vector fragment of pSG1448/27/95/21/4

- 5 (pSG144angAIIangAIIangorfI4angMIII). The ligation was used to transform *E. coli* DH10B with standard procedures and plasmid pSG1448/27/95/21/4eryK (pSG144angAIIangAIIangorfI4angMIIIeryK) is isolated. The construct is confirmed with restriction digests.

10 *Isolation of plasmid pSG1448/27/95/21/4eryK4/19*

Plasmid pSGLit24/19 from *E. coli* ET12567 is digested with *Xba*I and the insert fragment is isolated and ligated with the *Xba*I treated vector fragment of plasmid pSG1448/27/95/21/4eryK. The ligation is used to transform *E. coli* DH10B with standard procedures and plasmid pSG1448/27/95/21/4eryK4/19

- 15 (pSG144angAIIangAIIangorfI4angMIIIeryKangB) is isolated. The construct is confirmed with restriction digests.

Isolation of plasmid pSG1448/27/95/21/4eryK4/193/6eryCIII

Plasmid pSG1448/27/95/21/44/193/6eryCIII is digested with *Bgl*II and the about 2.1 kb

- 20 fragment is isolated and ligated with the *Bgl*II treated vector fragment of pSG1448/27/95/21/4eryK4/19. Plasmid pSG1448/27/95/21/4eryK4/193/6eryCIII is isolated using standard procedures and the construct is confirmed using restriction digests. The plasmid is used to transform mutant strains of *S. erythraea* with standard methods.

25 *Bioconversion of 3-O-mycarosyl erythronolide B to 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A*

The *S. erythraea* strain Q42/1pSG1448/27/95/21/4eryK4/193/6eryCIII is grown and bioconversions with fed 3-O-mycarosyl erythronolide B are performed as described in the General Methods. The cultures are analysed and a compound with m/z 750 is detected

- 30 consistent with the presence of 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A.

Example 8: Production of 13-desethyl-13-methyl-5-O-mycaminosyl erythromycins A and B; 13-desethyl-13-isopropyl-5-O-mycaminosyl erythromycin A and B; 13-desethyl-13-secbutyl-5-O-mycaminosyl erythromycin A and B

Production of 13-desethyl-13-methyl-3-O-mycarosyl erythronolide B, 13-desethyl-13-isopropyl-3-O-mycarosyl erythronolide B and 13-desethyl-13-secbutyl-3-O-mycarosyl erythronolide B

Plasmid pLS025, (WO 03/033699) a pCJR24-based plasmid containing the DEBS1, DEBS2 and DEBS3 genes, in which the loading module of DEBS1 has been replaced by the loading

5 module of the avermectin biosynthetic cluster, was used to transform *S. erythraea*

JC2ΔeryCIII (isolated using techniques and plasmids described previously (Rowe *et al.*, 1998; Gaisser *et al.*, 2000)) using standard techniques. The transformant JC2ΔeryCIIIpLS025 was isolated and cultures were grown using standard protocols. Cultures of *S. erythraea*

10 JC2ΔeryCIIIpLS025 are extracted using methods described in the General Methods section and the presence of 3-O-mycarosyl erythronolide B, 13-desethyl-13-methyl-3-O-mycarosyl erythronolide B, 13-desethyl-13-isopropyl-3-O-mycarosyl erythronolide B and 13-desethyl-13-secbutyl-3-O-mycarosyl erythronolide B in the crude extract is verified by LCMS analysis.

15 *Production of 13-desethyl-13-methyl-5-O-dedesosminyl-5-O-mycaminosyl erythromycin A and B, 13-desethyl-13-isopropyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A and B, 13-desethyl-13-secbutyl-5-O-dedesosminyl-5-O-mycaminosyl erythromycin A and B*

Cultures of *S. erythraea* JC2ΔeryCIIIpLS025 are extracted using methods described in the General Methods section and the crude extracts are dissolved in 5 ml of methanol and subsequently fed to culture supernatants of the *S. erythraea* strain

20 SGQ2pSG1448/27/95/21/44/193/6eryCIII using standard techniques. The bioconversion of 13-desethyl-13-methyl-3-O-mycarosyl erythronolide B, 13-desethyl-13-isopropyl-3-O-mycarosyl erythronolide B and 13-desethyl-13-secbutyl-3-O-mycarosyl erythronolide B to 13-desethyl-13-methyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A and 13-

25 desethyl-13-methyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B; 13-desethyl-13-isopropyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A and 13-desethyl-13-isopropyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B; 13-desethyl-13-secbutyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A and 13-desethyl-13-secbutyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B is verified by LCMS analysis.

30 **Example 9: 13-desethyl-13-methyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A and 13-desethyl-13-methyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B**

Production of 13-desethyl-13-methyl-3-O-mycarosyl erythronolide B

Plasmid pIB023 (Patent application no 0125043.0), a pCJR24-based plasmid containing the DEBS1, DEBS2 and DEBS3, was used to transform *S. erythraea* JC2ΔeryCIII using standard techniques. The transformant JC2ΔeryCIIIpIB023 was isolated and cultures were grown using standard protocols, extracted and the crude extract was assayed using methods
5 described in the General Methods section. The production of 3-*O*-mycarosyl erythronolide B, and 13-desethyl-13-methyl-3-*O*-mycarosyl erythronolide B is verified by LCMS analysis.

Production of 13-desethyl-13-methyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin A, 13-desethyl-13-methyl-5-O-dedesosaminyl-5-O-mycaminosyl erythromycin B
10 Cultures of *S. erythraea* JC2ΔeryCIIIpIB023 are extracted using methods described in the General Methods section and the crude extracts are dissolved in 5 ml of methanol and subsequently fed to culture supernatants of *S. erythraea* SGQ2pSG1448/27/95/21/44/193/6eryCIII using standard techniques. The bioconversion of 13-desethyl-13-methyl-3-*O*-mycarosyl erythronolide B to 13-desethyl-13-methyl-5-*O*-
15 dedesosaminyl-5-*O*-mycaminosyl erythromycin A and 13-desethyl-13-methyl-5-*O*-dedesosaminyl-5-*O*-mycaminosyl erythromycin B are verified by LCMS analysis.

Example 10: Production of 5-*O*-dedesosaminyl-5-*O*-mycaminosyl azithromycin
20 Azithromycin aglycones were prepared using methods described in EP1024145A2 (Pfizer Products Inc. Groton, Connecticut). The *S. erythraea* strain SGT2pSG142 was isolated using techniques and plasmid constructs described earlier (Gaisser *et al.*, 2000). Feeding experiments are carried out using methods described previously (Gaisser *et al.*, 2000) with the *S. erythraea* mutant SGT2pSG142 thus converting azithromycin aglycone to 3-*O*-mycarosyl
25 azithronolide. Biotransformation experiments are carried out using *S. erythraea* SGQ2pSG1448/27/95/21/44/193/6eryCIII and crude extracts containing 3-*O*-mycarosyl azithronolide are added using standard microbiological techniques. The bioconversion of 3-*O*-mycarosyl azithronolide to 5-*O*-dedesosaminyl-5-*O*-mycaminosyl azithromycin is verified by LCMS analysis.
30

Example 11: Production of 5-*O*-dedesosaminyl-5-*O*-mycaminosyl erythromycin C

Isolation of the S. erythraea mutant SGPI (SGQ2ΔeryG)

To create a chromosomal deletion in *eryG*, construct pSGΔG3 was isolated as follows:

Fragment 1 was amplified using primers BIOSG53 5'-
GGAATTCGGCCAGGACGCGTGGCTGGTCACCGGCT -3' and
BIOSG54 5'-GGTCTAGAAAGAGCGTGAGCAGGCTTCTACAGCCAGGTCA -3' and
genomic DNA of *S. erythraea* was used as template. Fragment 2 was amplified using primers
5 BIOSG55 5'-GGCATGCAGGAAGGAGAGAACGATGACCACCGACG-3' and
BIOSG56 5'-GGTCTAGACACCAGCCGTATCCTTCTCGGTTCCCTTGTG-3' and
genomic DNA of *S. erythraea* was used as template. Both DNA fragments were cloned into
SmaI cut pUC19 using standard techniques, plasmids pUCPCR1 and pUCPCR2 were isolated
and the sequence of the amplified fragments was verified. Plasmid pUCPCR1 was digested
10 using EcoRI/XbaI and the insert band DNA was isolated and cloned into EcoRI/XbaI digested
pUC19. Plasmid pSGΔG1 is isolated using standard methods and digested with SphI/XbaI
followed by a ligation with the SphI/XbaI digested insert fragment of pUCPCR2. Plasmid
pSGΔG2 is isolated using standard procedures, digested with SphI/HindIII and ligated with
the SphI/HindIII fragment of pCJR24 (Rowe *et al.*, 1998) containing the gene encoding for
15 thiostrepton resistance. Plasmid pSGΔG3 is isolated and used to delete *eryG* in the genome of
S. erythraea strain SGQ2 using methods described previously (Gaisser *et al.*, 1997; Gaisser *et*
al., 1998) and the *S. erythraea* mutant SGP1 (SGQ2Δ*eryG*) is created.

Production of 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin C

20 The *S. erythraea* strain SGP1 (*S. erythraea* SGQ2Δ*eryG*) is isolated using standard
techniques and consequently used to transform the cassette construct
pSG1448/27/95/21/44/193/6*eryCIII* as formerly described. The *S. erythraea* strain
SGP1pSG1448/27/95/21/44/193/6*eryCIII* is isolated and used for biotransformation as
described in Example 2 and assays are carried out as described above to verify the conversion
25 of 3-O-mycarosyl-erythronolide B to 5-O-dedesosaminyl-5-O-mycaminosyl erythromycin C
by LCMS analysis.

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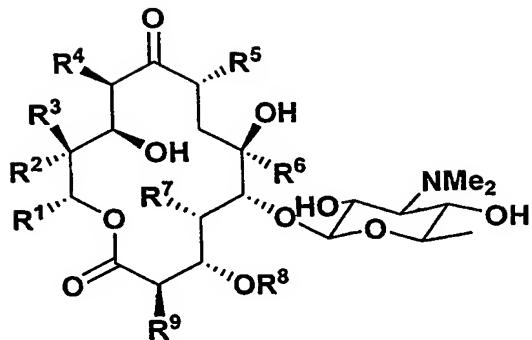
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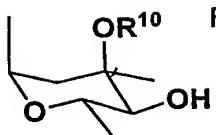
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Figure 1A

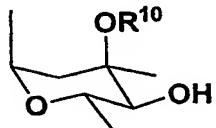
5-O-dedesosaminyl-5-O-mycaminosyl-erythromycin B

R¹= C₂H₅ R²= R⁴= R⁵= R⁶= R⁷= R⁹= -CH₃ R³= -H R⁸=

R¹⁰= CH₃

5-O-dedesosaminyl-5-O-mycaminosyl-erythromycin A

R¹= C₂H₅ R²= R⁴= R⁵= R⁶= R⁷= R⁹= -CH₃ R³= -OH R⁸=

R¹⁰= CH₃

5-O-dedesosaminyl-5-O-mycaminosyl-erythromycin C

R¹= C₂H₅ R²= R⁴= R⁵= R⁶= R⁷= R⁹= -CH₃ R³= -OH R⁸=

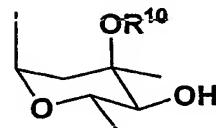
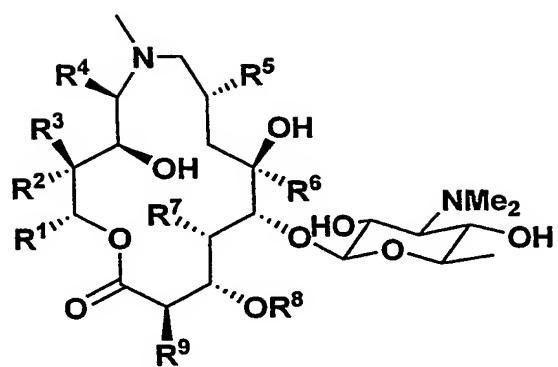
R¹⁰= H

Figure 1B



5-O-dedesosaminyl-5-O-mycaminosyl-azithromycin

$$R^1 = C_2H_5 \quad R^2 = R^4 = R^5 = R^6 = R^7 = R^9 = -CH_3 \quad R^3 = -OH \quad R^8 = \boxed{\text{---}} \quad OR^{10} \quad R^{10} = CH_3$$

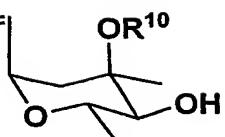


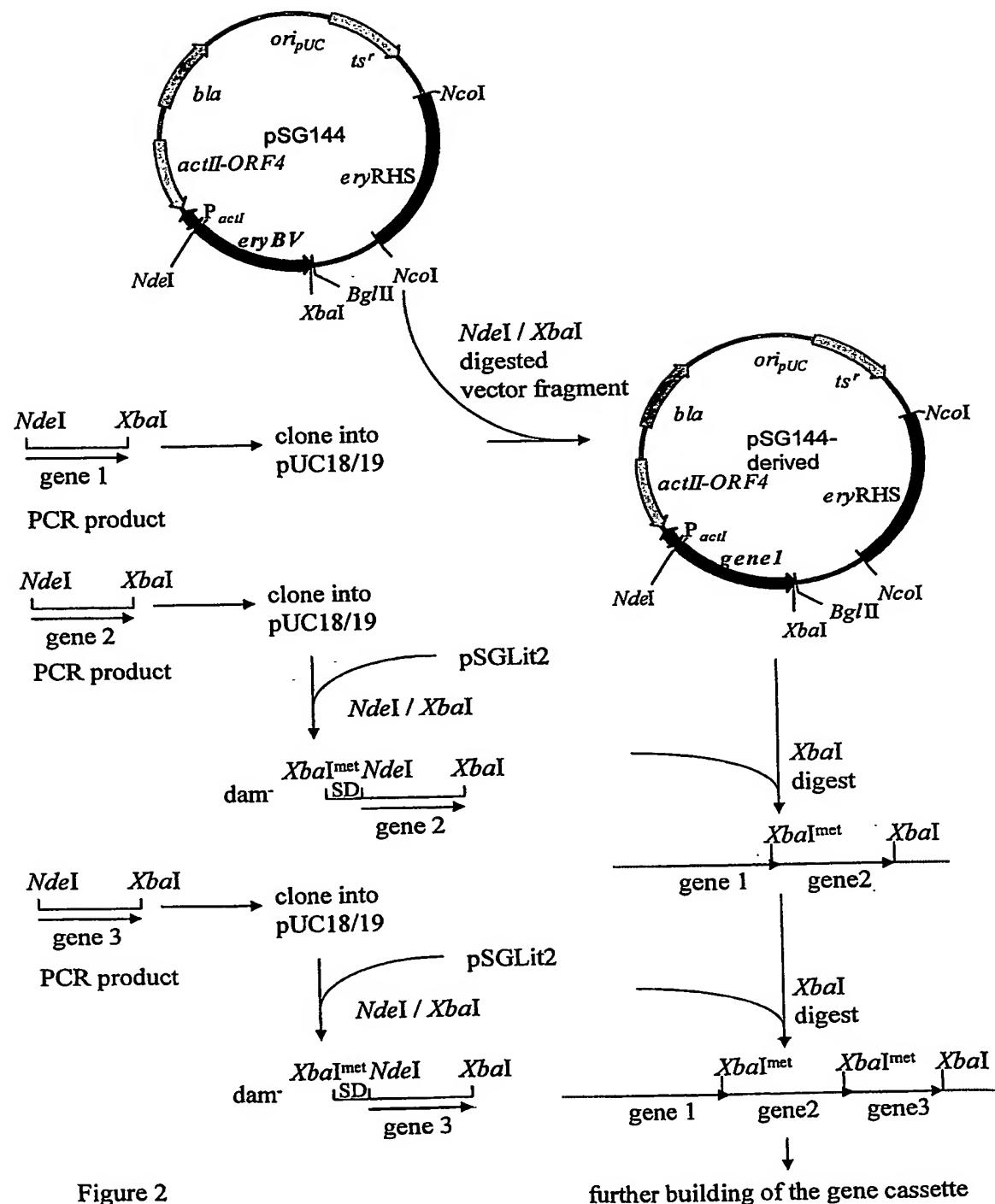
Figure 2

Figure 2

Figure 3

TylA1.pep x u08223.em_pro2

1 MNDRP~~RR~~RAMKGII~~LAGGSGTRLRPLTGTLSQ~~LLPVYDKPMIYYPLS~~VLM~~ 50
1 MNDRP~~RR~~RAMKGII~~LAGGSGTRLRPLTGTLSQ~~LLPVYDKPMIYYPLS~~VLM~~ 50
51 LAGIREIQI~~ISSKDHL~~D~~FRS~~LLGEGDRLGLSISYAEQREPRGIAEAFLI 100
51 LAGIREIQI~~ISSKDHL~~D~~FRS~~LLGEGDRLGLSISYAEQREPRGIAEAFLI 100
101 GARHIGGDDAALILGDNVFHGPGFSSVLTGTVARLDGCELFGYPVKAHR 150
101 GARHIGGDDAALILGDNVFHGPGFSSVLTGTVARLDGCELFGYPVKAHR 150
151 YGVGEIDSGGRLLSLEEKPRR~~PRSNL~~AVTGLYLYTNDVVEIARTISPSAR 200
151 YGVGEIDSGGRLLSLEEKPRR~~PLEP.~~GRHRLYLYTNDVVEIARTISPSAR 199
201 GELEITDVNKVYLEQGRARAR~~I~~TELGRGFAWLDMGTHDSLLQAGQYVQLLEQ 250
200 GELEITDVNKVYLEQGRA.AHGAGAVVAWLDMGTHDSLLQAGQYVQLLEQ 248
251 RQGERIACIEEIAMRMGFISAEQCYRLGQELRSSSYGSYIIDVAMRGAAA 300
249 RQGERIACIEEIAMRMGFISAEQCYRLGQELRSSSYGSYIIDVAMRGAAA 298
301 DSRAQ 305
299 DSRAQ 303

Figure 4

TylAII.pep x u08223.em_pro2

1 MRVLVTGGAGFIGSHFTGQLLTGAYPDLGATRTVVLCKLTYAGNPANLEH 50
||| ||| ||| ||| |||
1 MRVLVTGGAGFIGSHFTGQLLTGAYPDLGATRTVVLCKLTYAGNPANLEH 50

51 VAGHPDLEFVRGDIADQALVRRLMEGVGLVVHFAAESHVDRSIESSEAFV 100
||| ||| ||| |||
51 VAGHPDLEFVRGDIADHGWWRRLMEGVGLVVHFAAESHVDRSIESSEAFV 100

101 RTNVEGTRVLLQAAVDAGVGRFVHISTDEVYGSIAEGSWPEDHPIAPNSP 150
||| ||| ||| |||
101 RTNVEGTRVLLQAAVDAGVGRFVHISTDEVYGSIAEGSWPEDHPVAPNSP 150

151 YAATKAASDLLALAYHRTYGLDVRVTRCSNNYGPROYPEKAVPLFTTNLL 200
||| ||| ||| |||
151 YAATKAASDLLALAYHRTYGLDVRVTRCSNNYGPROYPEKAVPLFTTNLL 200

201 DGLPVPLYGDGGNTREWLHVDDHC RGVALVAAGGRPGVIYNIGGGTELTN 250
||| ||| ||| |||
201 DGLPVPLYGDGGNTREWLHVDDHC RGVALVGAGGRPGVIYNIGGGTEL TN 250

251 AE^LTDRI^LELCGADR^SA^VRRVADRP^GHDR^RYSVDTTKI^REELGYAPRTGI 300
||| ||| ||| |||
251 AE^LTDRI^LELCGADR^SALRRVADRP^GHDR^RYSVDTTKI^REELGYAPRTGI 300

301 TEGLAGTVAWYRDNR^AWWEP^LKRSPGGRELERA 333
||| ||| ||| |||
301 TEGLAGTVAWYRDNR^AWWEP^LKRSPGGRELERA 333

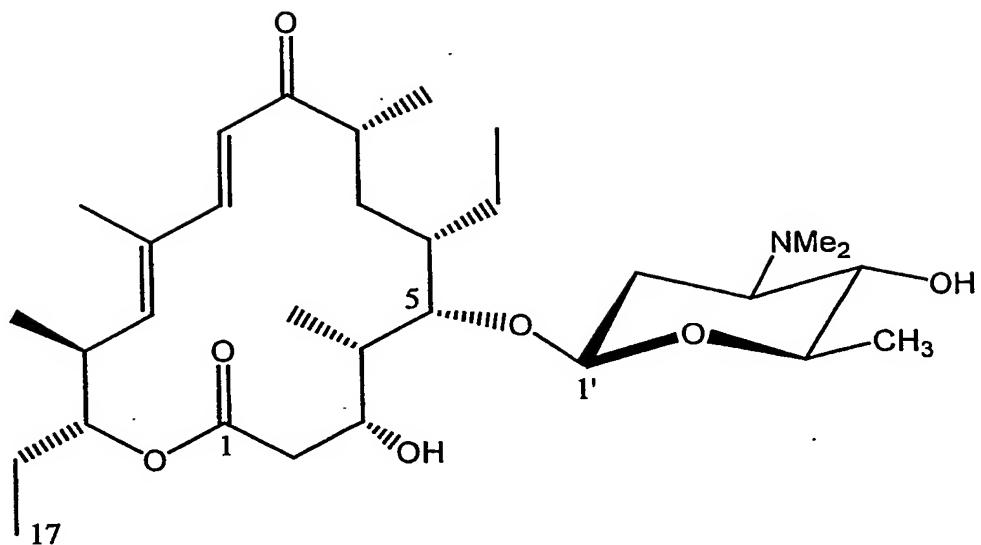
Figure 5

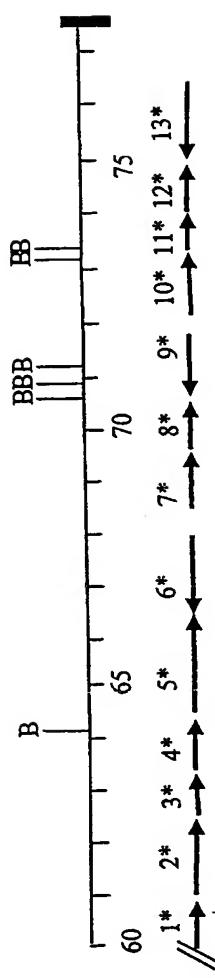
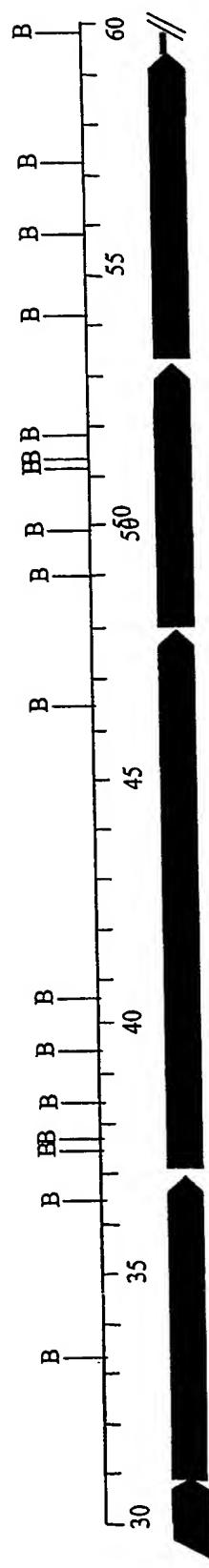
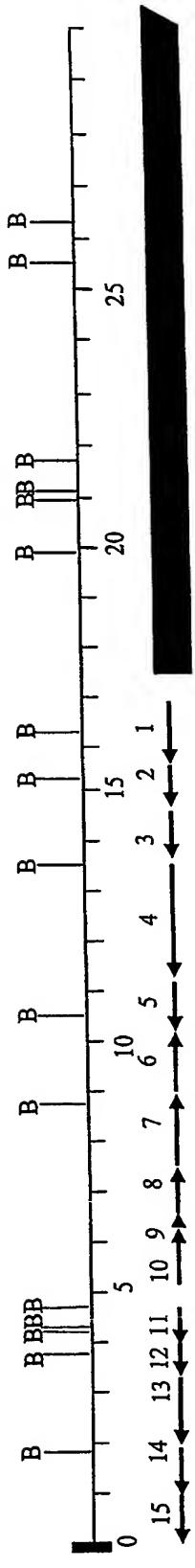
Figure 6

Figure 7

1 GGCATGCCTT CGGGGTGTGC GGCGGCGCCT CAGAGCGTGG CCAGTACCTC
51 GTGCAGGGCC GCGATCACCT TGTCCGTAC GTCGGGCGCG AGCCCCGGGT
101 ACATCGGCAG CGAGAAAGATC TCGTCGCCA GCCGCTCCGT CACCAGCAGC
151 GAGCCCTTGG CGTACCCCAG GTGCGCGAAG CCCGTATGG TGTGCACGGG
201 CCACGGGTAA CTGATGTTGA GCGAGATCCC GTACGACTTG AGCGCCTCGA
251 TGATGTCGTC CCGGCGCGGG TGGCGGACGA CGTACACGTA ATACACGTGG
301 TCGTTGCCCT CGGTGACGGA CGGCAGCACC AGGCCGCCGG GGCCCGTCAG
351 GTTCGCGAGT CCTTCGGCGT AACGCCGGC GACCGCGCGC CGGCCCTCGA
401 TGTAGCGGTC GAGGCGGGTG AGCTTGCGGC GCAGGATCTC CGCCTGCACC
451 TCGTCGAGCC GGCTGTTGTG GCCGGCGTC TGCACGACGT AGTACACGTC
501 CTCCATGCCG TAGTAGCGCA GCCGGCGCAG CGCACGGTCG ACGTCCCGT
551 CGTCGGTCAG CACGGCCCCG CCGTCGCCGT ACGCACCGAG GACCTTCGTC
601 GGGTAGAACG AGAAGGCGGC GGCGTCGCC AGCGTGCAGG CCAGCTCGCC
651 GTGGTGGCGG GCACCGTGCG CCTGGCGCA GTCCCTCCAGC ACCACCAGGC
701 CGTGCTGCTC GGCCAGGGCG CGCAAGGGCG CCATGTCGAC GCACTGCCCG
751 TACAGGTGCA CCGGCAGCAG GGCCTCGTG CGCGGGGTGA TGACGTCCGC
801 GACCTGGTCG GTGTCCATGA GGTGGTCCTC GGCGCGGACG TCGACGAAGA
851 CGGGCGTGGC ACCGGTGCCG TCGATGGCCA CCACCGTCGG CGCGGCCGTG
901 TTGGAGACGG TGACGACCTC GTCCCCCGGG CCCACCCCGA GCGCCTGCAG
951 ACCCAGCTTG ACGGC GTGGTGCAGA TGCCGTTGTC GACACCGCCG CAGTGGCGCA
1001 GGCGTGGTA GTCCGCGAAC TCCTTCTCGA ACCCGTCCAC GCTGGGGCCG
1051 AGGACCAACT GCCCGGAGGC GAAGACGGTC TCGACGGCGT CGAGGAGGTC
1101 CGCGCGTTCG TTCTGGTATT CCGCCAGGTA GTCCCAGACG TAGGTAGTCA
1151 CGGAGAGCTC AACCTCCAGA GTGTTCGAT GGGGTGGTGG GAAGCCGGTG
1201 CGCGCGGACC AGGTCGTGC AGCAGTCGCG GACCGACTCC CGCAGCGAAC
1251 GGCGCGGTGC CCAGCCCAGC AGGGCGCGCG CCGCGCCGGT GTCGACCCGC
1301 AGCCAGTCCT CCCGGTGCCCC GGGAGCCCGG CCCGGAGCCG GCGCCTCCAC

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1351 CACCCGCGCC GGAATGCCGC TCGCCTCGAT GAACAGGCCG ACCAGGT CGC
1401 GGACGGCGAC CGCCTCGCCC CGCCCGATGC CGACGGCGAC CGGGACGGCC
1451 GGTGCGCGGG CGGC GGCCAC GACGGCGTCG GCCACGTCCC GCACATCGAC
1501 GTAGTCCCGG TGCGCGCGCA GCCGGGACAG TTCCACGACG GCCTCCGCAC
1551 CCGTCCCGGC GGCGGCCAGC AGCCGCTCGG CGACCTGGCC CAGCAGACTG
1601 ATCCGCGGGG TGCCGGGGCC CGACACGTTG GACACCCGTA GCACCACACC
1651 GTCGACCCAC CCGCCCCGAGG TGCCCCGAG CACCGCCTCG CTGGCGGCGA
1701 GCTTGCTCCT GCCGTACGCC GTGTCCGGC GCGGTACGGC GTCGGCGCCC
1751 ACCGAACCGC CGGGCGTCAC CGGGCCGTAC TCCAGTACCG AGCCGAGGTG
1801 GACCAGCCGC GGCGCGCGG ACATCAGCGC CAGCGCCTCC AGCAGGCGCA
1851 GCGTGGGCAC CGCGGTGGCG GACCACATCT GCTCGTCGGT ACGGCCCCAG
1901 ATGCTTCCGA CGGAGTTGAC GATCGTGTCC GGACGCTCCG CGTCCAGGGC
1951 GGCGGCCAGC GCCGCGGGAT CCGTACCGGC CAGGTCCAGG GTGACGCAGC
2001 GGTACGGCAT CGGCTCCTCG GGCGGGCGGC GGCCCACAC CACCACGTCA
2051 CGGCCCCGCG CGCGAACGC CGCGCACACA TGCCGGCCGA CGTACCCGGC
2101 GCCGCCAGG ACCACGACGC TGCCACTGCC ACTGCCGCAC GGCATCGGAT
2151 CGTTCACCAT

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Figure 8

11301 CGTCAGTACA GCGTGTGGGC ACACGCCACC AGGGTGCAGA GCTCGATGTT
11351 GAGGTAGTTG CCGTGCGCCA GCAGCCCAGT GAGCTGACCG AGCGACAGCC
11401 AGGCGAAGTC GTCCGGTGCG TCCTCCGGGA AGTCGTGCGG GACCTCCACG
11451 ATCACGTAGC GGTTCTGGGC GTGGAAGAAG CGCCCGCCCT CCTCGGACTG
11501 GACGGCGTCG TAGCGCACGT CCTGAGGCAGG CGCGGACAGC ACGTCCTCCA
11551 GGTACGGCGG GCCGGGCAGC CCCCGCGGAC CGGTGTGCTC CTGTGGCCGG
11601 CACTGGACCG TGGGGGCCAG CTCGGCGACG TTCAGGTGCC CGACGTCCAC
11651 CCGTGCCCCGC ACGAGCGCGT GCAGCACGCC GTCGACGGAC TTGACCAGCA
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11751 GTGACCTCCC GGCTGCTGGC GCTGACCTCG GCGGCCATGA CCCGGAAGTG
11801 CCGCCCCGTC TCGTGGCGA TCTCGTGCAG CGTGCAGGTAC CAGCCGTCCG
11851 CCGTCACCGT ATCGAGCGGC ACCCGGTTCT GCACCAGCTC CCGCAGGGCG
11901 CGCACACCCG TGAACCACGT CAGGACCTCG GCCGTCGTGT GCCGCGCCGC
11951 ACCCGGCGAG CCGAAGAAGG AGCGCAGCAC GGGGGACGGG GCGGACGCGT
12001 CGGCGTCCGC CGTGGGCAGG CAGGCAGGA TGGACCGGGC GTCCATGTTG
12051 ACCACGTTGT CCAGCATCAG CAGCCGGCGG AGCTGCCCA GCGTCAGCCA
12101 GCGGAAGTCC TCCCCGATGT CGAGGTGTC GTCCGCCGCC AACTCGACGA
12151 TCATGTTCCG GTTGCCTTG GCCAGGAACC AGTCCGCCTG TTCGGACTGG
12201 ATCGAGTCGA CCAGGACACG CGCCCGTCGC GGGCCCCATGA ACAGGTCCAG
12251 ATAGCGGATG TCGCGCCCCC GGTGCACCCC GGTGAAGTTG CTCCGGGTGG
12301 CCTGCACGGT CGGCGACACC TGAAGAACGT TGACGTTCCC GGGCTCCATC
12351 TTGGCCTGCA TCAGGAAGTG CAGCACCCCG TCGATCTCCC GCGCCACGAT
12401 CCCGAGCAGC CCCACCTCCG GCTGCACGAT GATGGGCTGC GTCCAGCCCC
12451 GCTCGGGCAG CGGGTCCGTA CGGACGTGCA GCCCCTCCAC GGAGAAGAAA
12501 CGGCCCGACG CGTGGTGCAG GTTCCCCGTA CCCGGGTGGA AGCTCCAGCC
12551 GCGCAGCTCC GCGAAGGGAA CGCGGGACAC GTCGAAGCGC CCCGCCCCGCA
12601 GGCGTTCGGC CAGCCAGCCG GAGATGCCGT CGAACGGCGT GACCGCACTG

12651 TCCGCGGTGC GTGCCGACAC CAGCACCCGC CGCGCCGTGT CCACCGGGTC
12701 ACCGGGCCGG ACCCGTCCG CACGGCGCCG CGCGGCCCG TGCGGGCGG
12751 GGGCGGATCG CGCGGTACG GGTCGCGGG CGGTGTCCCGC GGCGGTGCGC
12801 GGCAGGGACGG GGCGCGTGCT CGTGTCCGCG CGGGTACCGCG GTGGGACGGT
12851 CCCGGTGGCC GTGTCCGCGG TGGCGTGCC GGCGAGGGCG TCGCCGATGG
12901 TCCGGCACAC CTCGTCCATC CGGTGTTCA GATAGAAGTG ACCGCCGGCG
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14001 GAGCAGCCGC TTCGTCAAGCT CCCGGTTGGT CAGCTCCGTC CCGCCACCGA
14051 TGTGGTAGAC CTCGCCCGGG CGCCCGCGGG TCGCCACCAAG GCTGATCCCG

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14101 CGGCAGTGGT CGTCCACGTG CAGCCAGTCC CGGCTGTTGC CGCCGTCGCT
14151 GTACAGCGGC ACCGTCAGAC CGTCCAACAG GTTCGTGGCG AAGAGCGGGA
14201 CGACCTTCTC GGGGTGCTGG TACGGGCCGT AGTTGTTGGA GCACCGGGTG
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14351 CCTCGCGCCA CGACCCCTCG GCGATCGAGC CGTACACCTC GTCCGTGGAG
14401 ACGTGGACGA ACCGGCCGGC CCCCGCCTCC ACCGCGGCCT GCAAGAGGAC
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14501 AGCGGTCCAC GTGCGACTCC GCCGCGAAGT GGACCACGAC GTCCGCCCG
14551 CGCACGACCC GGGACATCAC CTCCGCGTCC CGGATGTCGG CGTGCACGAA
14601 CTCCAGCGAC GGATGGTCCG CGACCGGGTC CAGGTTGGCG AGGTTCCCGG
14651 CATAGGTCAG CTTGTCGACC ACCACCGTCC GCGCCCCGGC CAGGTCCCGA
14701 TACGCCCGG CCAGCAGTTG TCTGACGAAG TGCGAGCCGA TGAAGCCCGC
14751 ACCTCCGGTG ACCAGCAGCC GCATGGGAGC ACAGACCTT CTTCCAGGGA
14801 CGGGAAACGG GGAGGGCGGAC GGGGACGGAG GCGAGGGCGG TGGCTATGCG
14851 GCCGGTCCGG ACATGAGGGT CTCCGCCACG TCCATCAAGT ACCGGCCGTA
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14951 TGTACCCCAT CCGCAGGGCG ATCTCCTCGA CGCAGGAGAT CCGCACGCC
15001 TGCCGCTGCT CCAGGAGCTG GACGTACTGC CCCGTTGCA GCAGCGAGCT
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15401 AGACGTTGTC CCCCAGGATG AGGGCGACCG GGTCGTCCCC GATGTGCTCC
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15651 CGGCAGCAAC TGCTTGGACA GTGCCCGGT CAGGGGGCGC AGGCGCGTGC
15701 CGCTGCCGCC CGCCAGGATG ATGCCCTTCA TGGGCCGCCG GTCCGCCGTC
15751 GTCTTCGTCA T

Figure 9

59800	G
59801	TGAGCCCCGC ACCCGCCACC GAGGACCCGG CCGCCGCCGG GCGCCGCCCTG
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59901	CGCGCTCGTC CTGCGCGCCG AGGCCACCGA CCCGGCCCCG TACGAGGAGC
59951	GGATCCGGGC CCACGGGCCG CTCTTCCGCA GCGACCTGCT CGACACCTGG
60001	GTCACGGCGA GCAGGGCCGT CGCCGACGAA GTGATCACCT CACCCGCCCTT
60051	CGACGGGCTC ACGGCCGACG GGCGGCCGCCC CGGCGCGCGG GAACTGCCGC
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60301	CGCAGGCTCC CCGCGCTGGT CCTCCGCGAA CAGCTCGCG TGCGGGAGGA
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60401	ACGGCGCCCT GTGCCCGCAA CTCCCTCCGG ACGCCGTGGC GGGGGTGC
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60501	GGCGGGCCGG GCCCCCGACG CCGTCGCCGC CGCCCGCACC CTGGCGTCG
60551	CGGCGCCGA GCGCGAGCC ACCCTCGTCG GCAACGCCGT ACAGGGAGCTG
60601	CTGGCGCGTC CGCGCAGTG GCGGGAGCTC GTACGCGACC CGCGCCTCGC
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60851	CCCGTACCTC CGCCCCCTCC GTCCCTCGAG CCCCCCTCGA CCTCACACGG
60901	CCCGTGGCCG CGCCCGGGCC GTTCGGGCTC CCCGGCGACC TGCACCTCCG
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 61351 GAGATCGGCG ACGACCTCGT CCTCTACCAG CAGGGCATGG ACTTCGTGGA
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 62351 CCGACGAGAT GCTCGCCGCC CCCACCCCGG CCGCGCTCGT CCCCCGCCTC
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 62451 CATGAACCTC GAATACAGCG GCGACATCGC CCGGTTGTAC GACCTGGTCC
 62501 ACCAGGGAAA GGGCAAGGAC TACCGGGCGG AGGCCGAGGA GCTGGCCGCG

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62751 CCGCTTCGAC GCCGTGATCT GCATGTTCAAG TTCCATCGGG CACATGCGCG
62801 ACCAGCGGGA ACTGGACGCG GCGATCGGCC GGTTCGCCGC GCACCTGCCG
62851 TCCGGCGGGG TCGTGATCGT CGATCCCTGG TGGTTCCCGG AGACGTTCAC
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63001 GACTACCTCG TCGGCGTGCC GGGGGAGGGG GTGCGGCACT TGAAGGAGAC
63051 CCATCGGATC ACGCTTTCG GGCGTGCAGA GTACGAGGCG GCCTTCACCG
63101 CGGCAGGGAT GTCCGTGAG TACCTCCCGC ACGCCGCCAC CGACCGCGGA
63151 CTCTTCGTCG GCGTCCAGGC CTGA

Figure 10

1 MKGIILLAGGS GTRLRPLTGA LSKQLLPVYD KPMIYYPLSV LMLAGIRDIQ
51 IITSKTHLEM FRSLLGDGSR IGISVGYAEQ EEPRGIAEAF LIGEEHIGDD
101 PVALILGDNV FHGPGFSSVL ASTAARLDGC ELFGYPVKDP RRYGVGEVDA
151 EGRLVSLEEK PEKPRSHLAV TGLYFYDNGV VDIARRLTPS PRGELEITDV
201 NKVYLEQGRA RMTELGRGFA WLDMGTHSSL LQAGQYVQLL EQRQGVRISC
251 VEEIALRMGY ISARQCHELG RELESSSYGR YLMDVAETLM SGPA

Figure 11

1 MRLLVTGGAG FIGSHFVRQL LAGAYPDLAG ARTVVVDKLT YAGNLANLDP
51 VADHPSLEFV HADIRDAEVM SRVVRGADV VHFAAESHVD RSIADASAFV
101 ETNVRGTQVL LQAAVEAGAG RFVHVSTDEV YGSIAEGSWR EEQPLAPNSP
151 YAASKAASDL LALAYHRTYG LPVVVTRCSN NYGPYQHPEK VVPLFATNLL
201 DGLTVPLYSD GGNSRDWLHV DDHCRGISLV ATRGRPGEVY HIGGGTELTN
251 RELTKRLLGL CGADASSVRH VADRPGHDLR YALDIGKITG ELGYAPRTDF
301 TTGLADTVRW YAENRAWWEP LKKAAQEARR TD

Figure 12

1 VSTPSAPPVP GAPSPAGHPD EGLWVRRYRP VRDPELRLVC FPHAGGAATS
51 FAALARGLDE TVEALAVQYP GRQDRRHEPF IPSISGLVDQ VVPEILRWAD
101 RPLALFGHSM GATVAFEVAR RLRGSGQASP VHLLVSGRRA PTVRRRDVAH
151 LLDDDALIAE IATLQGTEDA VLQDEELLRL ALPAIRNDYR AAGTYAYVPG
201 GALDCPVTVL TGDRDPDVPL EEARAWRELT TGPFALHTFA GGHFYLNDRM
251 DEVCRTIGDA LAGTATAADTA TGTVPVRTAA DTSTGPVPPR TAADTAREPV
301 PPRSAPAPHG AARRRADAVR PGDPVDTARR VLVSARTADS AVTPFDGISG
351 WLAERLRAGR FDVSRVVPFAE LRGWSFHPGT GNLHHASGRF FSVEGLHVRT
401 DRLPERGWTQ PIIVQPEVGL LGIVAREIDG VLHFLMQAKM EPGNVNVLQV
451 SPTVQATRSN FTGVHRGRDI RYLDLFMGPR RARVLVDSIQ SEQADWFLAK
501 RNRNMIVELA ADDDLDIGED FRWLTLGQLR RLLMLDNVVN MDARSILACL
551 PTADADASAP SPVLRSSFFGS PGAARHTTAE VLTWFTGVRA LRELVQNRVP
601 LDTVTADGWY RTPHEIAHES GRHFRVMAAE VSASSREVTS WTQPLIEPRL
651 PGLMALLVKS VDGVLHALVR ARVDVGHLNV AELAPTVQCR PQEHTGPRGL
701 PGPPYLEDVL SAPPQDVRYD AVQSEEGGRF FHAQNRYVIV EVPHDFPEDA
751 PDDFAWLSLG QLTGLLAHGN YLNIELRTLV ACAHTLY

Figure 13

1 MVNDPMRGS GSGSVVVLGG AGYVGRHVCA AFAARGRDVV VVGRRPPEEP
51 MPYRCVTLDL AGTDPAALAA ALDAERPDTI VNSVGSIWGR TDEQMWSATA
101 VPTLRLLEAL ALMSARPRLV HLGSVLEYGP VTPGGSVGAD AVPRPDTAYG
151 RSKLAASEAV LRGTSGGWVD GVVLRVSNVS GPGTPRISLL GQVAERLLAA
201 AGTGAEAVVE LSRLRAHRDY DVVRDVADAV VAAARAPAVP VAVGIGRGEA
251 VAVRDLVGLF IEASGIPARV VERPAPGRAP GHREDWLRVD TGAARALLGW
301 APRRSLRESV RDCWHDLVRA HRLPTTPSKH SGG

Figure 14

1 VTTYVWDYLA EYQNERADLL DAVETVFASG QLVLGPSVDG FEKEFADYHG
51 LRHCGGVNDNG TNAVKLGLQA LGVPGDDEVV TVSNTAAPTV VAIDGTGATP
101 VFVDVRAEDH LMDTDQVADV ITPRTKALLP VHLYGQCVDM APLRALAEQH
151 GLVVLEDCAQ AHGARHHGEL AGTLGDAAAAF SFYPTKVLGA YGDGGAVLTD
201 DADVDRALRR LRYYGMEDVY YVVQTPGHNS RLDEVQAEIL RRKLTRLDRY
251 IEGRRAVARR YAEGLANLTG PGGLVLPST EGNDHVYYVY VVRHPRRDDI
301 IEALKSYGIS LNISYPWPVH TMTGFAHLGY AKGSLPVTER LADEIFSLPM
351 YPGLAPDVQD KVIAALHEVL ATL

Figure 15

1 VSPAPATEDP AAAGRRLQLT RAAQWFAGTQ DDPYALVLRA EATDPAPYEE
51 RIRAHGPLFR SDLLDTWVTA SRAVADEVIT SPAFDGLTAD GRRPGARELP
101 LSGTALDADR ATCARFGALT AWGGPLLPAH HERALRESAE RRAHTLLDGA
151 EAALAADGTV DLVDAYARRL PALVLREQLG VPPEAATAFE DALAGCRRTL
201 DGALCPQLLP DAVAGVRAEA ALTAVLASAL RGTPAGRAPH AVAAARTLAV
251 AAAEPAATLV GNAVQELLAR PAQWAELVRD PRLLAAAATVE TLRVAPPVRL
301 ERRVARED TD IAGQRLPAGG SVVILVAAVN RAPVSAGSDA STTVPHAGGR
351 PRTSAPSVPSP APFDLTRPVA APGPFGGLPGD LHFRLLGGPLV GTVAEEAALGA
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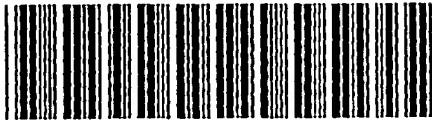
Figure 16

1 MRILLTSFAH NTHYYNLVPL GWALRAAGHD VRVASQPSLT GTITGSGLTA
51 VPVGDDTAIV ELITEIGDDL VLYQQGMDFV DTRDEPLSWE HALGQQTIMS
101 AMCFSPNLD STIDDMVALA RSWKPDVLW EPFTYAGPVA AHACGAAHAR
151 LLWGPDVVLN ARRQFTRLA ERPVEQREDP VGEWLTWTLE RHGLAADADT
201 IEELFAGQWT IDPSAGSLRL PVDGEVVPMR FVPYNGASVV PAWLSEPPAR
251 PRVCVTLGVS TRETYGTDGV PFHELLAGLA DVDAEIVATL DAGQLPDAAG
301 LPGNVRVVDF VPLDALLPSC AAIVHHGGAG TCFTATVHGV PQIVVASLWD
351 APLKAHQLAE AGAGIALDPG ELGVDTLRGA VVRVLESREM AVAARRLADE
401 MLAAPTPAAL VPRLERLTAH HRRA

Figure 17

1 MNLEYSGDIA RLYDLVHQGK GKDYRAEAEAE LAALVTQRRP GARSLLDVAC
51 GTGMHLRHLG DLFEEVAGVE MSPDMLAIAQ RRNPEAGIHR GDMRDFALGR
101 RFDAVICMFS SIGHMRDQRE LDAAIGRFAA HLPSGGVVIV DPWWFPETFT
151 PGYVGASLVE AEGRTIARFS HSALEDGATR IDVDYLVGVP GEGVRHLKET
201 HRITLFGRAQ YEAAFTAAGM SVEYLPHAAT DRGLFVGVQA

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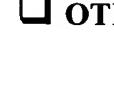
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